Synopsis for the Submission of the Proposed Thesis Entitled

EVALUATION OF ANTIMUTAGENICITY AND ANTICANCER ACTIVITIES OF *ASPERGILLUS TERREUS*

By

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Ph.D registration No. MU/EXB/Ph.D./CR.57/Biosc./2015-16/
E.13, Dated : 03.01.2014.

In Fulfilment of the Requirement for the Award of

Doctor of Philosophy

in Biosciences

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MAY 2022

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ABSTRACT

Filamentous fungi are some of the most exploited microbial resources for their various structurally diverse metabolites, possessing potential activities such as antimicrobial, anticancer, and antioxidant properties. Even though numerous studies have been conducted on the biological and industrial potential of fungal metabolites, only a few percent of the fungal constituents have been identified and exploited in the field of drug discovery in cancer research. In this scenario, a study was conducted to evaluate the possible antioxidant, antimicrobial, antimutagenic, and anticancer properties of some selected fungi, such as *Aspergillus terreus*, *Aspergillus niger*, and *Penicillium citrinum*, employing *in vitro*, *in vivo*, and *in silico* models.

The ethyl acetate extracts of all the selected fungi were found to be rich in phytochemical compounds such as steroids, saponins, alkaloids and phenolics. The results of the antioxidant and antimutagenicity evaluations showed that the fungi, *A. terreus*, possess significant antioxidant properties and are non-mutagenic to the bacterial system. Furthermore, *A. terreus* extracts were found to be antimutagenic/protective against cyclophosphamide and sodium azide-induced mutagenicity in bacterial (*Salmonella typhimurium*) and plant (*Allium cepa*) models.

The results of the influence of physicochemical parameters highlighted that temperature, pH, salinity, and different culture media constituents played a vital role in the optimal biomass and secondary metabolite production of *A. terreus*. Furthermore, the fungi were found to possess antimicrobial properties against certain pathogenic microorganisms. *In vitro* cytotoxicity evaluations of the fungal extract on cancer cell lines showed that the extract exhibited significant cytotoxicity in HeLa cell lines and was non-toxic to fibroblast cell lines. *In vivo* experiments on zebrafish (*Danio rerio*) embryos revealed that *A. terreus* extract possesses the potential for inducing apoptosis and anti-angiogenesis. The chemical compounds in the ethyl acetate extracts of *A. terreus* were identified by FT-IR and GC-MS analysis. The in silico molecular docking studies of the identified compounds against certain HeLa cell proteins revealed that some compounds possess a strong protein-ligand interaction, suggesting they may be good drug candidates for cancer therapy. Thus, among the selected fungus, the ethyl extract of *A. terreus* was found to possess antioxidant, antimicrobial, antimutagenic, and anticancer properties. A multidisciplinary approach to the exploitation of natural resources, especially fungal metabolites, would be the best option to overcome the limitations and crisis in the field of cancer drug discovery and development.

1. Introduction and Review of Literature

1.1. Introduction

Microorganisms are rich sources of bioactive compounds such as antibiotics, industrial enzymes, antioxidants, and other pharmacologically important molecules. Numerous studies indicate that among all the microbes, fungi, and actinomycetes have been found to produce a vast variety of bioactive metabolites (Gunatilaka, 2006). These bioactive molecules are generally referred to as secondary metabolites, which are diverse in structure and function and range from intracellular or extracellular small molecules to larger bio-molecules including proteins, polysaccharides, or their complex forms (Demain and Fang, 2000). Because fungi are highly specific in their nutritional and growth parameters, it is possible to induce the production of a wide range of biomolecules by changing these parameters (Fernandes *et al.*, 2009).

Antioxidant compounds from various fungi have contributed immensely to the discovery of novel and potent drugs (Sharma *et al*., 2016). The majority of them are used in pharmaceuticals as drug candidates, but none have been approved as anticancer agents so far. Despite the fact that a significant amount of research has been conducted on the utilization of fungal metabolites in various fields of the economy, only a few research studies have highlighted the importance of fungal metabolites as promising bioactive molecules in cancer drug discovery (Ameen *et al.,* 2021).

Compounds derived from natural sources account for approximately 60% of all drugs under clinical trials, and some of them are being confronted in cancer drug discovery as a rich source of active therapeutics (Qurishi *et al*., 2011). Various approaches, including the use of natural products, are being used to develop epigenetic-based targeted therapies. Small molecules derived from natural products that induce non-apoptotic cell death and cell viability can help to solve the problem of multidrug resistance. Thus, the exploration of fungi for bioactive molecules is important in this context because they are one of the most common groups of organisms with a wide range of secondary metabolites.

Antitumor drugs, often known as anticancer agents interact with rapidly dividing cells to prevent or delay the formation and progression of tumors. Bioactive metabolites produced by microorganisms and plants have various ways of inhibiting tumor growth. These metabolites have anti-tumor and anti-mutagenic properties. Antimutagenic agents work to reverse the

effects of mutagens. Anti-tumor drugs stop cancer cells from multiplying by inducing apoptosis or necrosis (Penta, 2016).

Due to the technical challenges associated with the mass cultivation of fungi and the isolation of adequate quantities of metabolites, the search for natural products in cancer drug discovery of fungal origin still has been restricted (Linnakoski *et al*., 2018). The advancement of new strategies and techniques in the field of natural product research can cope with these difficulties and bio-molecules of fungal origin could contribute significantly in the area of cancer drug research.

With this background information and worldwide high demand for new anticancer and antimutagenic agents, and the fungal species with a variety of bioactive molecules, the present work was carried out to explore the possible antimutagenic and anticancer activities of certain fungal extracts. It was aimed to carry out a detailed study selecting one among the fungal extracts which exhibited the highest activity.

The study was carried out with the following objectives:

- To culture certain selected fungi and prepare the crude extracts with different polar and non-polar solvents.
- To screen the fungal extracts for various constituents.
- To evaluate the fungal extracts for antimutagenicity against a broad-spectrum anticancer drug.
- To conduct a study on growth parameters and antimicrobial properties of the selected fungi possessing antimutagenic activity.
- To evaluate the fungal extracts for anticancer activity using *in vitro* and *in vivo* assays.
- To conduct a detailed study on the fungal extract that exhibited the maximum anticancer activity.

1.2. Review of Literature

1.2.1. Natural Products-A Continuing Source of Novel Drugs

Natural products of plant and animal origin have been widely used as an important source of components for traditional medicine preparation to treat a variety of diseases since ancient times (Cragg and Newman, 2013). The phytochemicals or natural product extracts are high in biologically active compounds and have the potential to cure many diseases or act as functional

foods. Many of the currently used chemotherapeutics are derived from nature, either as a component or as the entire product. Comprehensive reviews by Newman and Cragg (2012) highlighted the critical role of natural products as a rich source of molecules in cancer therapy. Currently, 131 of the 175 cancer therapeutics in use are non-synthetic and are either natural products or derivatives of them; so much so, even today, natural compounds are at the forefront of cancer chemotherapeutic combinatorial libraries (Qurishi *et al.,* 2011; Huang *et al*., 2021).

A plethora of natural products, including molecules derived from microorganisms have been found to possess the property of scavenging the free radicals thereby ameliorating the mutagenicity and carcinogenicity of certain agents (Masuma *et al.*, 2001). Thus, natural product chemistry is an emerging trend and has received worldwide attention in the area of therapeutic research and development.

1.2.2. Role of Fungi in Addressing Major Global Challenges

It has been advocated that fungi are one of the potential bioactive sources for addressing major global challenges of culminating the health disorders, including cancers (Kumar *et al*., 2021). However, fungal diversity has not yet been widely used because only a small percentage of them are grown in laboratories for commercial applications. Despite the fact that fungal metabolites are structurally diverse, including phenolics, aromatic compounds, amino acids, anthracenones, aliphatic compounds, naphthalenones, pyrones, and terpenes, none of the fungal derived compounds have been approved as anticancer drugs (Rappe and Giovannoni, 2003; Demain, 2006; Kumar *et al*., 2021). In the present scenario, fungi are an inevitable groupof microorganisms in the field of natural product research, as they are the major resources of bioactive exploited for sustainable global development (Ameen *et al*., 2021).

1.2.3. Secondary Metabolites of Fungi

Different species of *Aspergillus*, *Penicillium*, and *Rhizopus* are known as the supreme chemical factories of nature. A vast variety of bioactive compounds such as mycotoxins, antifungal and anticancer agents have been isolated from these groups of filamentous fungi for the last 100 years (Frisvad *et al.,* 2004). The secondary metabolic machinery *of Aspergillus* species is a rich source of novel drug candidates and other industrially relevant enzymes and bio-molecules (Storz, 2005). The fungus is well-known for producing lovastatin, a secondary metabolite that is used as a drug to lower blood cholesterol levels. Earlier studies indicated

that *A. terreus* is a rapid producer of many bioactive molecules such as terpenes, statins, antifungals, anticancer agents, and polyketides (Masuma *et al*., 2001; Harsha *et al.*, 2013; Boruta and Bizukojc, 2016; Bhargavi *et al*., 2016). Sulochrin, terretonin, asterriquinone, and butyrolactone terreineol, terreulactone A, terrain, terreic acid, and aspulvinones are some of the biologically important compounds isolated from this fungus (Yamamoto *et al.*, 1980; Kim *et al*., 2002; Macedo *et al*., 2004).

Saprophytic *Penicillium* sp. and *Aspergillus* sp. are among the most popular members of the Eurotiales that live on the dead and decayed matter in nature. *A. niger*, the primary abundant ascomycete found within the soil surroundings has also been the rich source of many bioactive compounds and industrial enzymes. Various groups of intracellular or extracellular hydrolytic and oxidative enzymes involved in the breakdown of plant lingo-cellulose aid fungi in their role as soil saprophytes (Nierman and Nelson, 2002). It is also known as the common fungi associated with post-harvest spoilage of fruits, vegetables, and nuts. It is broadly referred to as a citric acid producer, additionally a significant model organism for research areas including the investigation of eukaryotic protein secretion, the impacts of different environmental factors on stifling or triggering of different biomass-degrading catalysts, fermentation process development, and so on (Nadumane *et al*., 2016).

1.2.4. Mutagens and Antimutagens of Natural Products

A chemical or physical agent that is capable of inducing change within the genetic material of an organism is referred to as ‘mutagen’. As chemical mutagens induce mutations by completely different mechanisms, many categories of them like alkylating agents, base analogs, and intercalating agents may be distinguished. Mutagens are classified as direct or indirect mutagens based on their interaction with genetic material (Zhang *et al*., 2005; Ferguson and Denny, 2007). Previously there are many studies revealing that several antitumor compounds act through the antimutagenic mechanism (Tsai *et al.*, 1996; Dion *et al*., 1997; Ikken *et al.*, 1999, Sloczynska *et al*., 2014). The search for antimutagenic compounds reflects the dynamically developing research in cancer diagnosis and therapy (Ferguson and Philpot, 2008; El-Sayed and Hussin, 2013).

Most mutagens affect cells by producing reactive oxygen species (ROS), which antimutagens neutralize before they enter the cell (Shay *et al*., 2009). Several studies have found that antioxidants use reactive oxygen species removal as a strategy, implying that

antioxidant and antimutagenic properties are intertwined (Lee *et al*., 2011; Tian *et al.*, 2012; Unal *et al.*, 2013; El-Souda, 2021).

1.2.5. Mutation and Cancer

Factors responsible for mutation can be external including chemical and physical agents called mutagens. Mutations can also occur as a result of errors in DNA replication, repair, and recombination. Based on the base pairs involved in this process, the outcome of a mutation or DNA damage in a cell can be lethal, neutral, negative, or sub-lethal. Germ line mutations can be passed down through generations, whereas somatic mutations can result in the occurrence of mutation-induced disorders such as cancer **(**Basu, 2018). There are many reports proving the close link between mutation and cancers (Moolgavkar and Knudson, 1981; Martincorena and Campbell, 2015; Brett *et al.*, 2021).

1.2.6. *In vitro* Models in Cancer

In vitro tumor models are important tools in cancer research, helping in identifying cancerous agents, developing cancer therapeutics, screening anticancer drugs and providing a low-cost screening platform for understanding the mechanism of tumor metastasis and the action of drug candidates (Chambers *et al*., 2002). Breakthroughs in tumor cell biology and 3D cell culturing, precision medicine, and targeted drug therapy have facilitated the rapid development of new *in vitro* tumor models with increased complexity and a wider range of output parameters, moving beyond simple cytotoxicity, cell invasion, and progression screening procedures (Steeg, 2006; Wirtz *et al*., 2011).

1.2.7. *In silico* Models in Cancer

Molecular docking is the process by which the target and ligand molecules bind in three dimensions. *In silico* docking studies are now used as a preliminary step in the field of drug discovery from natural products because the target-ligand binding affinity, bonding energy, and orientation of a molecule can predict its effectiveness as a drug candidate. As a result, molecular docking studies are an important step in the screening of novel bioactive molecules against challenging diseases such as cancer (Balunas and Kinghorn,2005). The advancement of experimental tools such as next-generation sequencing, microarrays, and spectroscopic

techniques made it much easier to assess the cancer genome, transcriptome, and proteome (Mardis, 2008).

1.2.8. Hypothesis of the Study

In the present study, three fungi, well- known for the production of various secondary metabolites, namely *Aspergillus niger*, *Penicillium citrinum* and *Aspergillus terreus* were selected. These fungi have been industrially and medically used for the past few years for the synthesis of many bioactive molecules. *A. terreus* is used for the synthesis of hypocholesterolemic agents such as statins, antifungals, and drugs for cardiovascular diseases. *A.niger* is a major producer of citric acid and various extracellular enzymes. *Penicillium citrinum* is considered the major producer of antibiotics, ergot alkaloids, diketopiperazines, polyketides, and immune suppressants (Harsha *et al.*, 2013; Boruta and Bizukojc, 2016).

Even though these fungal species are used for various purposes in different sectors, most of their biomolecules have not been identified or evaluated for their potential activity with special reference to antimutagenicity and anti-carcinogenicity. Hence, the present study was undertaken to evaluate the possible anti-mutagenic, cytotoxic and anticancer potential of these fungi by employing *in vitro* and *in vivo* experimental models.

2. Isolation and Culturing of the Selected Fungi and Preparation of Extract with Organic Solvents

2.1. Introduction

Filamentous fungi are a type of non-photosynthetic organism with a wide range of nutritional requirements. They rely on organic matter in their growth environment. Fungi are classified into different groups based on their nutritional needs, such as carbon, nitrogen, and energy sources. Laboratory culturing of fungi is difficult due to their nutritional heterogeneity, and growth and sporulation are dependent on physicochemical or external factors in the growth environment (Nevalainen *et al.*, 2014). Culturing of microbes in the laboratory involves the use of various growth media; known as culture media that are supplemented with nutrients. Molecular identification of fungi is currently gaining more importance as traditional phenotypic identification has encountered difficulty in identifying closely related species

(Balajee *et al*., 2009). The molecular methods of identification involve analysis of specific conserved regions in the genome and phylogenetic analysis of the DNA sequence information from multiple regions of ITS and coding proteins, which makes taxonomical classification ofthe fungi easier (Varga *et al*., 2005; Wagner *et al*., 2018).

2.2. Materials and Methods

The fungi under study were isolated from natural sources such as spoiled vegetables, and the extracts were made according to conventional techniques, as detailed below.

2.2.1. Culturing of the Selected Fungi

Aspergillus terreus was procured from MTCC, Institute of Microbial Technology; Chandigarh, India. *Penicillium citrinum* and *Aspergillus niger* were isolated from spoiled vegetables. The culturing and morphological observations were made by following the manuals of Pitt and Hocking (1997), McGinnis (2012) and authenticated with the help of taxonomists in the Department of Applied Botany, Mangalore University.

The culture was revived on potato dextrose agar (PDA) slants and stored at 4°C. To study the colony characteristics, the fungi were grown in PDA plates and observed for their color, texture, and pattern of growth, and other morphological features.

2.2.2. Molecular Identification and Phylogenetic Analysis of the Fungi

The PCR protocol, agarose gel electrophoresis, elution of the bands, DNA sequencing, and sequence analysis were performed according to the protocol by Prathibha *et al*. (2018). ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs were used to amplify the entire sequence of the 5.8S ribosomal RNA gene, I & II spacer regions, and partial sequence of the 18S ribosomal RNA gene and 28S ribosomal RNA gene areas of the organism (Cooke *et al*., 2000; White *et al*., 1990). A phylogenetic tree was constructed based on the ITS sequence of *Aspergillus* with other *Aspergillus species*, including various species sourced from GenBank, with the software MEGA–X version 10.2.0 (Kumar *et al*., 2018) using the neighbour-joining (NJ) method (Saitou and Nei, 1987) under 1,000 bootstrap replicates.

2.2.3. Mass Cultivation of the Selected Fungi and Preparation of Crude Extracts of Fungi

For the production of secondary metabolites, *A. terreus, P. citrinum and A. niger* were grown in liquid culture media through submerged fermentation. Since the fungi profusely grow in general culture media, PDB was used to culture them in laboratory conditions (Yadav *et al*., 2014; Bhattacharya *et al*., 2019). In the present study, the liquid cultures of fungi were mixed with three organic solvents, viz. n-Hexane, chloroform and ethyl acetate to obtain the crude fungal extracts. The fungi grown on PDB for 14 days were subjected to solvent extraction. The yield of dried extract was calculated and stored in a refrigerator (4^{o}C).

2.2.4. Statistical Analysis

The yield obtained was calculated and expressed as mean ± standard deviation. Statistical analysis was conducted by 2-way ANOVA followed by Dunnett's multiple comparison tests by using Graph pad Prism version 8.

2.3. Results

2.3.1. Isolation and Identification of the Selected Fungi

Aspergillus niger and Penicillium citrinum

In PDA, the initial stages of growth of *A. niger* showed whitish cottony mycelia which turned to black color after the conidial formation. The round black conidial spores gave the colony a black color, which had a powdery appearance all over the surface. A mature colony was observed to be round, dense, cottony, black on top and yellowish on the bottom. The edges of the matured colony were characterized by pale yellowish fissures. Microscopically, *A. niger* had septate filamentous hyphae with smooth conidiophores bearing black conidia. The conidia emerge radially, upheld by phialides. All these features were in accordance with Visagie *et al*.(2014).

P. citrinum exhibited moderately slow growth on potato dextrose agar at room temperature. The surface texture is soft and velvety to hairy (woolly tufts of soft hair). The colony appeared in grey-green color in the initial stages of growth and turned to greenish colonies on the emergence of spores. The converse side was light yellow to yellow-brown colored. *P. citrinum* was recognized by its greenish colonies and its spreading or simple conidiophores supporting phialides in brush-like groups known as penicilli. Hyphae were septate and conidiophores basic or extended (Visagie *et al*., 2014).

Aspergillus terreus

In PDA, the colonies of *A. terreus* were observed to be typically leathery and cinnamon-buff to sand brown in color with brownish exudates and dark brownish on the reverse side. The colonies were slightly elevated, and the colony edges were lighter in color. The conidia of *A.terreus* were thick, smooth and round with the conidial heads columnar, compact and biseriate. The smooth conidiophores were bearing small globosely pale yellow conidia.

2.3.2. Molecular Identification of the Fungi

The sequence with 635 bases of the ITS rDNA PCR product of MUR1 was determined and used as a query to search the GenBank DNA database using the BLAST search. It was found that there was 99.37% to identity with *Aspergillus terreus* strain DTO. The conformed sequence was deposited in the NCBI-GenBank database with accession number MW086575. A phylogenetic tree was constructed based on the ITS regions of the different *Aspergillus* spp. and other fungal sequences by the neighbor-joining (NJ) method. The tree indicated that the MUR1 is genetically closely related to *Aspergillus terreus* strain.

2.3.3. Mass Cultivation of the Selected Fungi and Preparation of Crude Extracts

The fungi actively producing secondary metabolites were indicated by the change in the color of the growth medium. *A. terreus* showed a color change from pale yellow to dark brown whereas *P. citrinum* imparted a light greenish color on the broth. The growth of *A.niger* in potato dextrose broth conferred a slightly yellowish color.

The cell-free culture filtrate was used for extraction with polar and non-polar solvents: n-Hexane, Ethyl acetate, and chloroform. A dark brown semi-solid crude extract was obtained after evaporation of the solvent and the yield was expressed as mg/100ml of filtrate. The extracts obtained were dried by exposed to air and the yield was calculated.

Among the three selected fungi, ethyl acetate extraction of *A. terreus* yielded the highest quantity of the crude extract (52.39±1.26 mg/100ml), followed by *P. citrinum* (42.52±0.96 mg/100ml) and *A. niger* (41.87±1.92 mg/100ml).

The solvent extraction with n-hexane yielded 29.28±0.06 mg/100 ml crude form in *P. citrinum*, followed by *A. niger (*21.3±0.07 mg/100 ml) and the yield of crude extract was least in *A. terreus (*19.35±1.86 mg/100 ml). On the other hand, the crude extract obtained with

chloroform was found to be 25.92±0.36 mg/100 ml in *A. terreus*, followed by *P. citrinum* (23.75±0.89 mg/ml), and the yield was lowest for *A. niger* (22.69±1.49 mg/100 ml).

2.4. Discussion

In the present study, it was noted that among the three fungi, *A. terreus* produced a high quantity of metabolites in all three solvents. As previously stated, *A. terreus* exhibited rapid growth in PDB, producing a thick mat of mycelia within seven days of incubation, and the rate of growth was the fastest when compared with those of the other two fungi.

The results showed that solvent extraction with n-Hexane yielded lesser yield in all three fungi, which could be attributed to the chemical nature of the compounds secreted by the fungi. N-Hexane is a non-polar solvent with a low boiling point that is commonly used to extract oil and other non-polar hydrocarbons. Thus, it is assumed that the fungal metabolites contain a very small percentage of oils and fats, which accounts for the low extract yield. Similar kinds of results have also been obtained in earlier studies (Fatima *et al*., 2015; Nakamura *et al*., 2017; Wakeel *et al*., 2019).

The findings were similar to those of Nawaz *et al.* (2020), who investigated the effect of solvent polarity on the yield and bioactive properties of *Phaseolus* beans. Razak *et al*. (2012) found that polar solvents are preferred for the extraction of biological molecules from plant leaves in terms of quantity and quality, which is consistent with our findings. Sharma *et al*. (2016) used methanol and ethyl acetate to evaluate secondary metabolites from some endophytic fungi, following a similar protocol. Gauchan *et al*. (2020) extracted the antioxidant compounds of endophytic fungi from Himalayan yew in ethyl acetate.

3. Qualitative Screening of the Selected Fungal Extracts

3.1. Introduction

Filamentous fungi are the repository for pharmaceutically important bioactive chemicals under the class phenolics, flavonoids, tannins, terpenes, and other important derivatives (Khalil *et al*., 2020). Due to the adverse side effects of chemically manufactured antioxidants, numerous studies have been conducted to investigate naturally occurring antioxidants in dietary supplements (Damiano *et al*., 2019; Ali *et al*., 2020), cosmetics (Boo, 2019; Vantran *et al*., 2019) and pharmaceuticals (Tungmunnithum *et al*., 2018; Anwar *et al*.,

2019). Among the bioactive constituents of fungi, phenolics are an important class of antioxidant phytochemicals that have received a lot of attention due to their antitumor, antimutagenic, antimicrobial, and anti-inflammatory properties (Rice-Evans *et al*., 1997; Liu *et al*., 2018; Zhu *et al*., 2019; Tako *et al*., 2020).

Assessment of antioxidant properties and identifying the antioxidant compounds in the fungal extracts is an alternative method in search of compounds exhibiting antimutagenic properties (Yen and Chen, 1995). The determination of antioxidant activity and the presence of phytochemicals in the extract is an easier method than carrying out antimutagenicity assessments. Hence, any antioxidant assay can be considered a preliminary analysis in the search for an antimutagenic or anti-carcinogenic compound. The antioxidant properties can vary among the fungal metabolites qualitatively and quantitatively depending on the chemical constitution of the extract. As antioxidant properties are attributed to phenolics, tannins, or other bioactive compounds, a phytochemical analysis along with any antioxidant assay is the most reliable method for the isolation and identification of compounds possessing antimutagenic properties. DPPH radical scavenging assay is the preliminary approach, rapid and simplest spectroscopic method for assessing the antioxidant potential of a compound, an extract or other biological sources (Ramos *et al*., 2003).

3.2. Materials and Methods

The bioactive compounds of the selected fungi were qualitatively screened, and the antioxidant activity of the fungal extracts was evaluated.

3.2.1. Phytochemical Screening of the Crude Extracts of the Selected fungi.

The fungal filtrate was subjected to preliminary phytochemical analysis for the presence of secondary metabolites such as alkaloids, amino acids, carbohydrates, tannins, phenolics, flavonoids, saponins and terpenes using standard protocols by, Parekh *et al*. (2006).

3.2.2. Antioxidant Assay: Determination of DPPH radical-scavenging activity

Antioxidant potential of the secondary metabolites was assessed by DPPH-free radicals scavenging activity as described by Yen and Chen (1995) with slight modification.

3.2.3. Total Phenolic Content

The total polyphenolics of ethyl acetate extracts of *A. terreus, A. niger*, and *P.citrinum* were determined by the Folin-Ciocalteu method as described by Singleton *et al*. (1999).

3.2.4. Statistical Analysis

The significance of variation in the total phenolic content among the different fungal samples was expressed as mean ± standard deviation and analyzed statistically by One Way ANOVA followed by Bonferroni's Multiple Comparison test.

3.3. Results

3.3.1. Phytochemical Analyses

The qualitative analysis of secondary metabolites in the culture filtrate was assayed for phytochemicals such as phenols, saponins, reducing sugars, alkaloids, tannins, terpenoids and steroids and the results are shown in table (1).

Table (1): Phytochemical constituents present in *A. terreus*, *P. citrinum* and *A. niger*

Chemical constituents	**Test performed**	**Fungal culture filtrate**		
		A. terreus	***P. citrinum***	***A. niger***
Terpenoids	Test with Chloroform &H_2SO_4	+	+	+
Phenolics	Lead acetate test	+	+	+
	Ferric chloride test	+	+	+
Saponin	Froth formation test	+	+	+
Tannins	Lead acetate test	+	+	+

Carbohydrates	Fehling'stest	+	+	+
	Benedict's test	+	+	+
	Barfoed's test	+	+	+
Alkaloids	Dragendroff's test	-	-	-
Steroids	Test with acetic anhydride H_2SO_4	-	+	+

3.3.2. Antioxidant Assay: Determination of DPPH radical scavenging activity

The crude ethyl acetate extract of *A. terreus* showed a significant DPPH scavenging activity (84.25±1.58%) against 0.1mM DPPH solution, followed by *P. citrinum* (61.56±0.99 %) and *A. niger* (57.17±2.5 %) respectively as presented in figure (1). The ethyl acetate extract of all selected fungi showed notable antioxidant activity compared to n-Hexane and chloroform extracts.

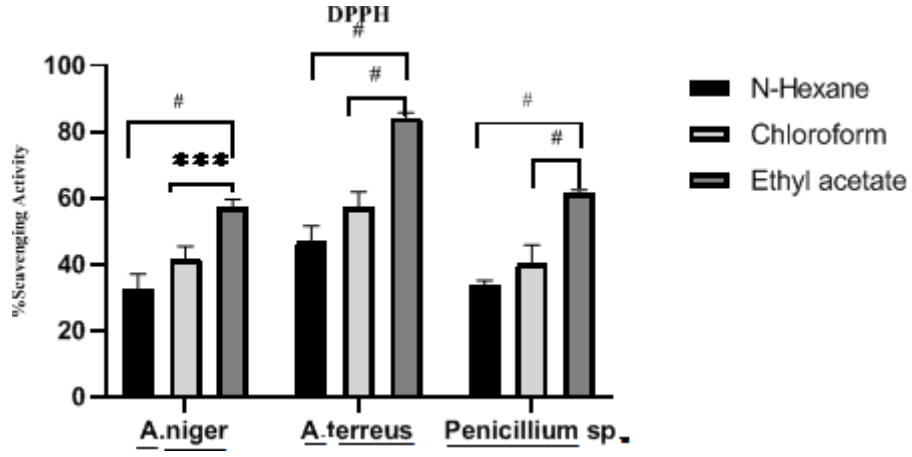


Fig (1): The percentage DPPH scavenging activity of secondary metabolites of the three selected fungi extracted with n-Hexane, chloroform and ethyl acetate.

Data was expressed as mean ± standard deviation; ***p<0.001; #p<0.0001

3.3.3. Total Phenolic Content

The total phenolic content determined by the Folin-Ciocalteu method was found to be 16.76±2.08µg/mg, 10.98±1.76µg/mg and11.8±0.85µg/mg for *A. terreus*, *A.niger* and *Penicillium citrinum* ethyl acetate extracts, respectively. The results indicate that the tested

extract is rich in polyphenols. Relatively, *A. terreus* was found to contain a higher amount of phenolics at a significant level compared to other extracts ($p < 0.05$).

3.4. Discussion

The cell-free aqueous filtrate of *A. terreus*, *P. citrinum* and *A. niger* showed the presence of various constituents such as phenolics, tannins, reducing sugars, saponins, alkaloids and terpenoids and exhibited significant antioxidant activities. Phenolics and terpenoids are the major chemical constituents responsible for a significant decrease in lipid peroxidation, a notable property of secondary antioxidants. The free radical scavenging property of phenols is credited to the presence of hydroxyl groups (Yadav *et al*., 2014). The antioxidant property of the fungal extracts may be attributed to their phytochemical constituents such as phenolics, saponins, terpenoids, steroids and alkaloids (Devi *et al*., 2012; Bhardwaj *et al*., 2015).

The current study is in parallel with an earlier report of Ibrahim *et al*. (2012), wherein ethyl acetate extracts of certain fungi exhibited higher equivalents of phenolics compared to other solvent extracts. The phenolic compounds present in the crude extract can be attributed to the radical scavenging property as evident from the two assays in the present study. A similar kind of observation was also made in an earlier study demonstrating the positive correlation between the antioxidant potential of *Aspergillus* species and total phenolic content (Arora and Chandra, 2011; Yadav *et al*., 2014).

Other than plants, fungi are also known to be the producers of saponins as their secondary metabolites. Saponins are the second most abundant phytochemicals that are widely used in the pharmaceutical industry. A number of previous literature reported that saponins have antioxidant (Cheng *et al*., 2011), anti inflammatory (Wang *et al*., 2013) and anticancer properties (Wu *et al*., 2012; Waheed *et al*., 2012).

Jin *et al*. (2017) evaluated the antimicrobial properties of saponins produced by two endophytic fungi from *Panax notoginseng*. According to the reports of Pan *et al*. (2017), the fungal endophytes that are isolated from the bulbs of *Fritillaria unibracteata* exhibited significant antioxidant activity which was contributed by phenolics, flavonoids and saponins. Dwi *et al*. (2019) showed that the ethyl acetate extract of *Aspergillus* sp. MFD-01 contained flavonoids, alkaloids, saponins, terpenoids, and tannins, which could explain its antioxidant activity.

Plants and animals are the primary producers of steroids, but are also produced by microorganisms (Sultan and Raza, 2015). Qin *et al*. (2009) isolated polyhydroxylated steroids from an endophytic fungus, *Chaetomium globosum*. Gao *et al*. (2011) evaluated the antifungal and cytotoxic activity of polyoxygenated steroids from the marine alga-derived endophytic fungus *Penicillium chrysogenum*.

Govindappa *et al*. (2011) found that *A. niger* and *Penicillium sp*. have strong antioxidant and anti-inflammatory activities, which they attribute to the high content of phenolics, tannins, saponins, and terpenoids in their extracts, which is consistent with our findings. Ethyl acetate extract of *A. terreus* isolated from *Ocimum sanctum* demonstrated antioxidant activity and a high phenolic content. *In vitro* studies of antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. by Yadav *et al*. (2014) revealed that the main phytochemicals present in endophytes were alkaloids, phenols, flavonoids, saponins, and terpenes, with the *Aspergillus* strain having the highest phenolic content and antioxidant activity.

The presence of hydroxyl groups in phenolics is thought to be responsible for their free radical scavenging ability. This study provided a glimpse into more in-depth research on bioactive compounds synthesized by fungi and its importance in the field of drug discovery.

4. Assessment of Antimutagenic Activity of the Selected Fungi

The antimutagenic activities of different compounds are assessed by using bacterial and plant models that have several advantages, including sensitivity, fast, and reliability. These assays are developed for screening a vast variety of chemicals and drugs for their potency in inducing DNA damage and thereby triggering mutation in cells (Issazadeh *et al*., 2012). The genotoxicity testing models are classified into groups based on the type of model organism and the level of mutagenicity detected. Prokaryotic testing systems typically evaluate DNA damage and gene mutations caused by a chemical agent, whereas eukaryotic bioassays can detect chromosomal damage and aneuploidies to a greater extent (Houk, 1992). In the present study, the anti-genotoxicity /antimutagenicity of the fungi were evaluated using the Ames test and *Allium cepa* mitotic aberration assay.

4.1. Assessment of Antimutagenicity-*Salmonella typhimurium* Reverse Mutation Assay

4.1.1. Introduction

The *S. typhimurium* (*Salmonella* test / Ames test) is a widely used short-term bacterial assay for detecting chemicals that induce mutations. A special strain of *S. typhimurium* which are auxotrophic to the amino acid histidine (his-) has been developed exclusively for the evaluation of gene mutations. The principle behind this assay is, that the histidine auxotrophic (his-) *Salmonella* strains are unable to grow and form colonies in the culture media that are not supplemented with histidine amino acid, as the mutations leave the bacteria unable to synthesize the amino acid by its own. When the mutant strains are exposed to a chemical mutagen, the bacteria undergo a second mutation which ultimately restores the ability of bacteria to synthesize histidine. The Ames test is also termed a 'reversion assay' as the second mutation revert the mutant his- strains to a revertant his+ strain.

4.1.2 Materials and Methods

Two mutant strains of the bacteria *Salmonella entrica typhimurium* (strain: TA 100; MTCC Acc. No. 1252, TA 98; MTCC Acc. No. 1251) were provided by Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH) Council of Scientific and Industrial Research (CSIR), Chandigarh, India. Sodium azide was used as the positive mutagen (Mortelmans and Zeiger, 2000).

4.1.2.1. Experimental Procedure: Pre-incubation assay (Ames *et al.,* 1977; Mortelmans *et al.,* 2000)

The test was conducted based on the plate incorporation method described by Maron and Ames (1983) using the bacterial strain. The ethyl acetate extract (6.25; 12.5; 25.0; 50.0 and 100.0 µg/plate dissolved in 0.05% DMSO) of *A. terreus, A. niger,* and *P. citrinum* were tested for mutagenicity. The fungi which did not exhibit any mutagenicity were further tested for their antimutagenic properties. The experiment was conducted in two ways: 1) Assessment of mutagenicity of the fungal extract; 2) assessment of antimutagenicity of the fungal extract. Sodium azide was used as the positive mutagen in both experiments. The mutagenicity was determined from the ratio between the number of His+ colonies formed in the test chemical

plate and the number of His+ colonies in the negative control plate. If the ratio was equal to or greater than 2, the experimental material was considered mutagenic (Ames *et al.*, 1977).

The results of the antimutagenicity test were calculated by using the following formula and as expressed as mutagenicity inhibition percentage (MIP) (Negi *et al.*, 2003):

$$MIP\ (\%) = (1 - T/M) \times 100\%$$

Where,

T - The number of revertant colonies per plate in the presence of mutagen and fungal extract, M - The number of revertants per plate in the presence of mutagen only without the fungal extracts.

The MIP results were divided into different categories to assess the protective effect of the fungal extracts. If the mutagenicity inhibition percentage (MIP) is between 0 and 25%, the extract is considered to have no antimutagenic effect; if the MIP range is between 25 and 40%, a moderate antimutagenic effect is assumed; and if the MIP value is greater than 40%, the sample is considered to exhibit a strong antimutagenic effect (Negi *et al.*, 2003).

4.1.2.2. Statistical Analysis

The experiments were conducted in triplicates and expressed as mean± SD. The data were analyzed by one way and two way ANOVA, $p<0.05$.

4.1.3. Results

4.1.3.1. Assessment of Mutagenicity

The results of assessment of mutagenicity of *P. citrinum* extracts were presented in figure (2). The *P. citrinum* extract treated with TA 100 strain showed a concentration dependant increase in number of revertant colonies with maximum number of His+ revertants were observed at a concentration of 100µg/ml. The fungi exhibited a similar pattern of growth in treatment with TA 98 strain, with maximal revertant colonies at a concentration of 100µg/ml. The concentration-response effect of *P. citrinum* extract on *S. typhimurium* TA 100 and TA 98 strains showed that the His+ revertant colonies were 2-3 folds and 3-5 folds higher than that

of the negative control in TA 100 and TA 98 strains respectively, when treated with the *P. citrinum* extract.

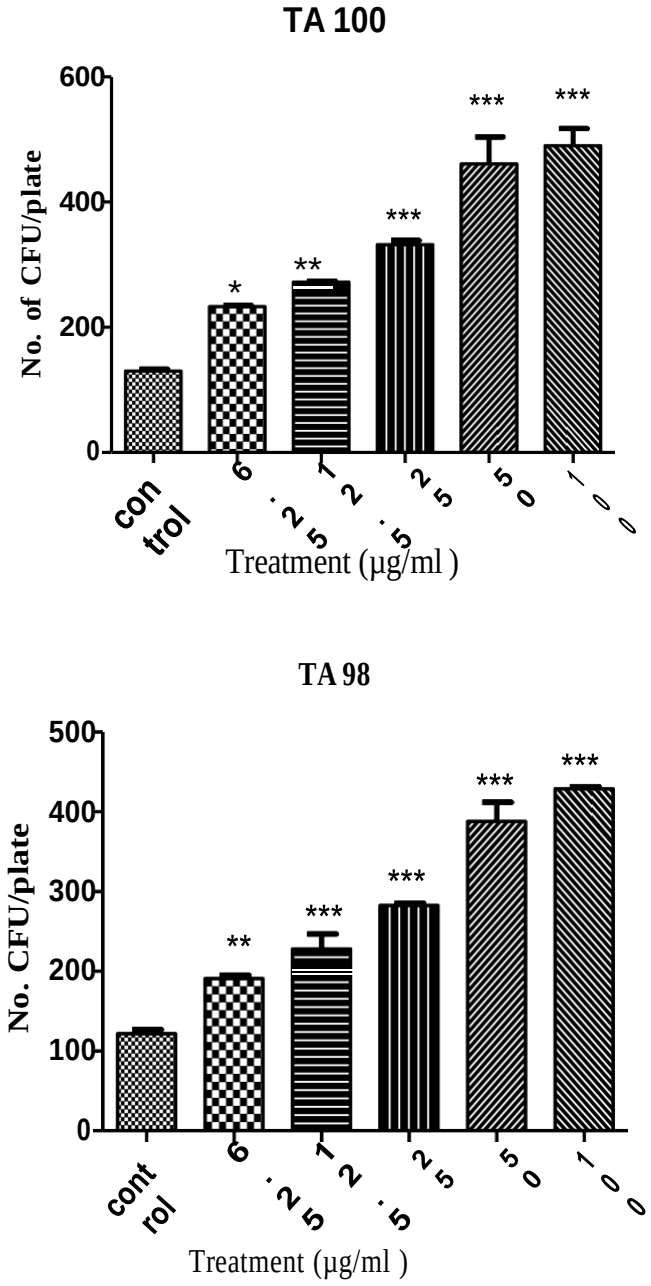


Fig (2): Effect of *P. citrinum* Extract on *S. typhimurium* TA 100 and TA 98 strains.

CFU: Colony forming unit; *: p<0.05; **p<0.001; ***p<0.0001

(Test sample Vs Solvent control).

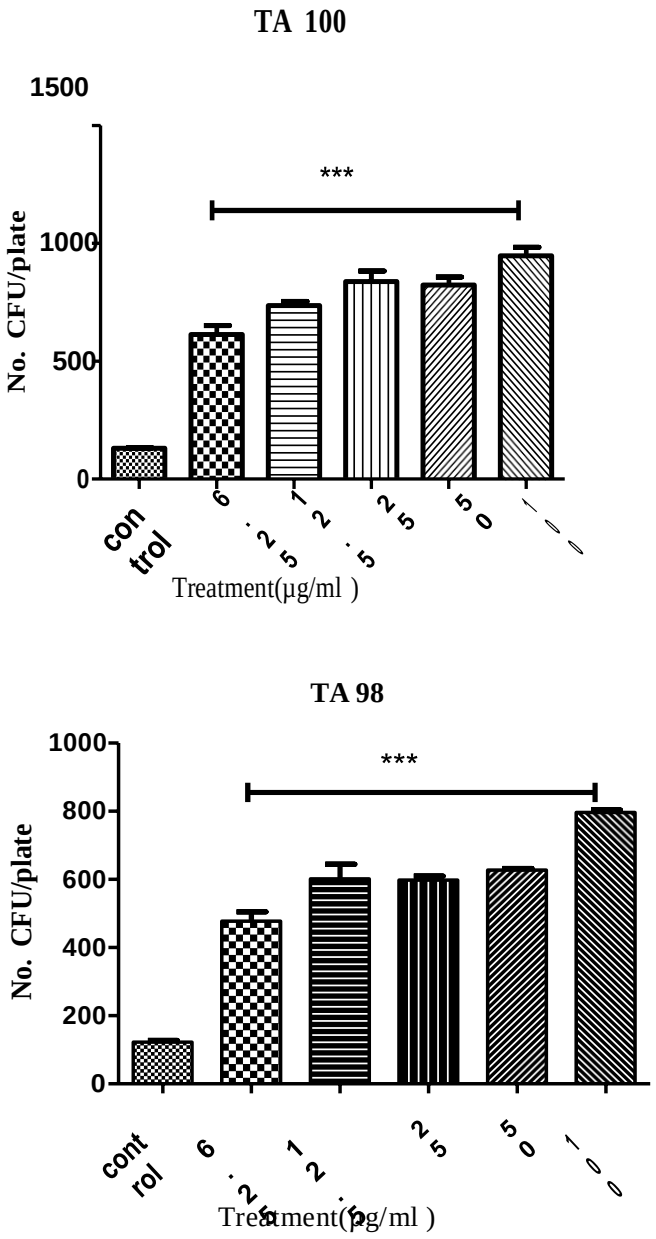


Fig (3): Effect of *A. niger* Extract on *S. typhimurium* TA 100 and TA 98 strains.

CFU: Colony forming unit. ***p>0.0001 (Test sample *Vs* Solvent control)

The ethyl acetate extract of *A. niger* exhibited similar pattern of genotoxicity as shown by *P. citrinum* extract, presented in figure (3). All the tested concentrations were found to be toxic against both TA 100 and TA 98 strains, with maximal number of revertant colonies were formed at 100µg/ml extract concentration. The increase in revertant colonies were calculated 4-7 folds in TA 100 and 7-9 folds in TA 98 when compared with the negative control.

The results of assessment of mutagenicity of *A. terreus* extracts were presented in fig. (4). The ethyl acetate extract of *A. terreus* treated with TA 100 showed an increase in the number of colonies with increasing the concentration, and the number of colonies was seen at

maximum in the plate treated with 50µg/ml. The slight decrease in the number of colonies at 100µg/ml concentration might be due to the antimicrobial effect of the extract at higher concentrations. This concentration dependant increase in the number of colonies was not statistically significant as the ratio between the extract-treated plates to the positive control plate was below 2. The fungi exhibited a similar pattern of growth in treatment with TA 98 strain; with the maximal colony forming unit was observed at a concentration of 100µg/ml.

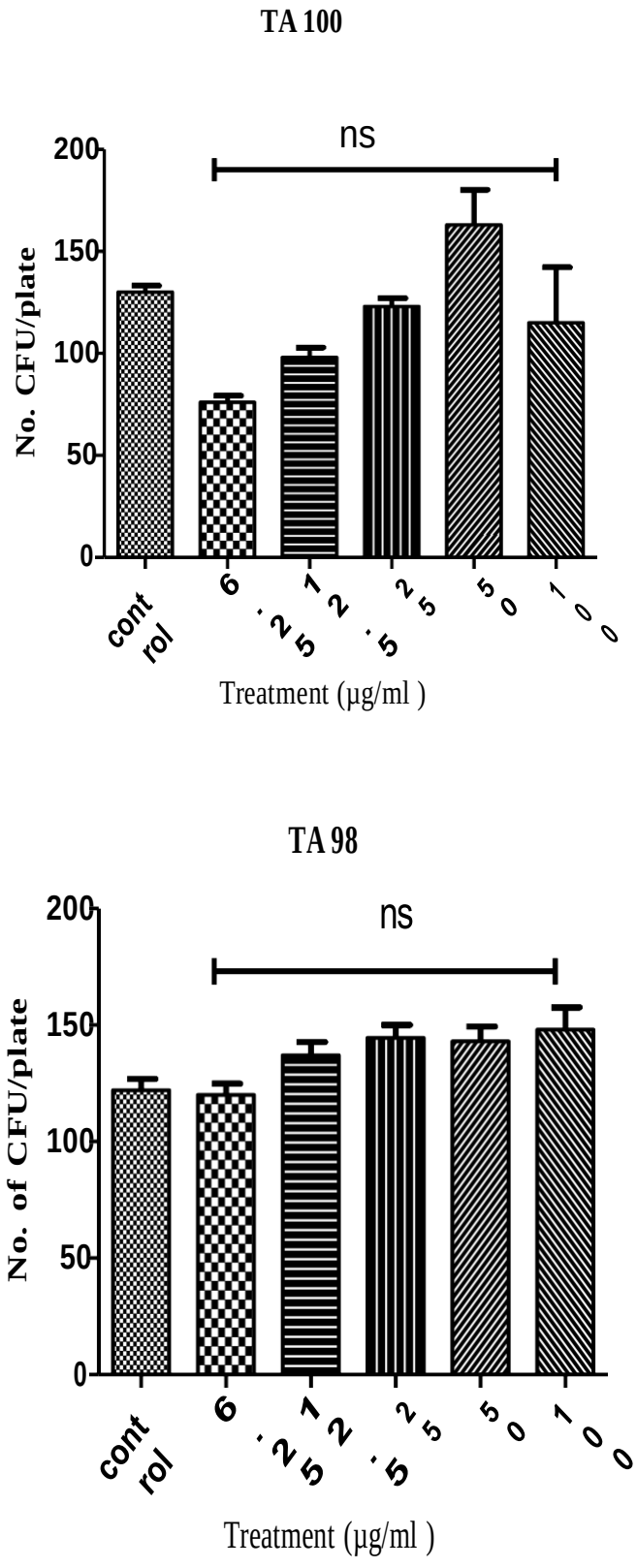


Fig (4): Effect of *A. terreus* Extract on *S. typhimurium* TA 100 and TA 98 strains.

CFU: Colony forming unit; ns: not significant (Test sample Vs Solvent control)

4.1.3.2. Assessment of Antimutagenicity

As the *A. terreus* extract did not exhibit mutagenic activity, its antimutagenic potency against a well-known mutagen was assessed and the results are presented in fig (4). The sodium azide (positive control) treated with different concentrations of *A. terreus* extract resulted in a significant reduction ($p<0.05$) in the number of colonies when compared to the plate treated with sodium azide alone in both TA 100 and TA 98 strains. Number of revertant colonies in the extract-treated plates was significantly lower than in the positive control ($p<0.05$).

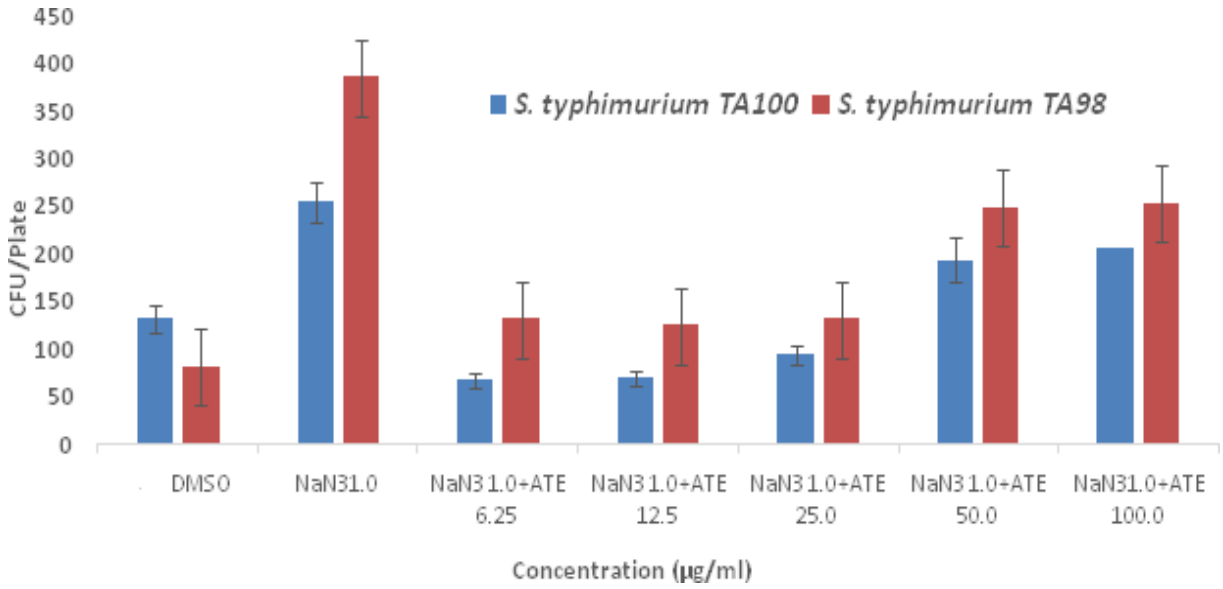


Fig (4): Antimutagenic activities of *A. terreus* against sodium azide (NaN_3)-induced mutagenicity in *S. typhimurium* TA 100 and TA 98 Strains; ($p<0.05$).

4.1.4. Discussion

The results of the mutagenicity assessment of the extracts of *A. niger* and *P. citrinum* showed that there was a significant increase in the revertant CFU/plate when compared to the number of colonies in the positive control plate alone. The number of revertant colonies (CFU/plate) of the bacteria treated with both the fungi was found to be 3 -9 folds increased when compared with the negative control. The increase in revertant colonies was found to be4-7 folds in TA 100 and 7-9 folds in TA 98 when compared with the negative control. It was previously reported that the Increased numbers of revertant colonies in test chemical treated plates compared to concurrent untreated control plates is an indication of the mutagenic potential of the test chemical (Samiei *et al*., 2015). Since TA 98 and TA 100 strains were exhibited a higher growth rate induced by the crude extracts, it was confirmed that the fungal extracts were able to induce frame shift mutation in TA 98 and base-pair substitutions in TA 100 strains. Hence, it was confirmed that the crude ethyl acetate extract of *A. niger* and *P.*

citrinum contains certain mutagenic compounds which are capable of inducing mutagenicity in bacterial mutagenicity/ genotoxicity assay.

On the other hand, the crude ethyl acetate extracts of *A. terreus* incorporated plates of *S.typhimurium* TA 100 and TA 98 stains did not show a significant increase in the His+ revertants/plate ($p<0.05$) on increasing the concentration. Hence it was concluded that the *A. terreus* extract did not induce any point mutations and are not capable of imparting toxicity to the bacterial cells. Various studies similar to the findings of our study were reported on the mutagenicity of natural products and chemical compounds had justified that the mutagenicity assessment by Ames test is a reliable *in vitro* methodology (Wehner *et al*., 1978; Lanier *et al*., 2020).

Sodium azide, a pesticide and heavy metal enzyme inhibitor in the metabolic pathways of living cells is proven directly acting mutagen in plants and animals. In plants and bacterial cells, the mutagenic potential of sodium azide is attributed to an intermediate amino acid analog L-azidoalanine [N_3-CH_2-CH (-NH_2)-COOH]. The synthesis of this intermediate compound is mediated by the enzyme O-acetylserine sulfhydrylase which acts as a biocatalyst in the addition of azide (N_3-)to O-acetylserine leads to the synthesis of azidoalanine (Sadiq and Owais, 2000). Furthermore, sodium azide induces chromosomal aberrations by producing direct mis-pairing during the excision repair mechanism of the cell. It was reported that the sodium azide produces base substitutions through A-T→G-C, G-C→A-T transition, and transversions (Olsen *et al*., 1993). Shiratori *et al*. (2017) and Lanier *et al*. (2020) reported that some species of *Penicillium* species isolated from rice grains and natural habitats were able to induce base-pair substitution in TA 100 strains, indicating its mutagenicity. Similar studies conducted by Wehner *et al*. (1978) confirmed that certain mycotoxins produced by *Penicillium* sp. and *Aspergillus* sp. is mutagenic to *S.typhimurium* TA 100 and TA 98 strains.

The *A. terreus* extracts showed a significant reduction in the histidine independent (His+) revertant colonies when combined with sodium azide in both the TA 100 and TA 98 testing systems ($p<0.05$). It can be interpreted that the fungal extract imparts protective effects against sodium azide-induced mutagenicity in the bacterial reverse mutation assay. Similar studies were reported by various researchers on the modulator effect of natural compounds against sodium azide in *S.typhimurium* mutagenicity assay (Gulluce *et al*., 2011; Issazadeh *et al*., 2012; Carneiro *et al*., 2017; Helvacioglu *et al*., 2021). Based on these findings, it can be interpreted

that the ethyl acetate extract of *A. terreus* does not act on the genetic material of bacteria. This is the first study of its kind to evaluate at the mutagenic and antimutagenic properties of *A. terreus* extracts.

4.2. Assessment of Antimutagenicity of A. terreus using Allium cepa

4.2.1. Introduction

Higher plants are regarded as model systems due to their sensitivity to mutations and the use of cells from various parts (roots, leaves, pollen) to monitor cellular damage ranging from point mutations to chromosomal aberrations (Fiskesjo, 1985; Grant, 1994). The *Allium cepa* test has been regarded as a low-cost and simple assay. One of the benefits of the test is that complex chemicals can be directly exposed to the plant system without any prior treatment, such as concentration extraction, etc. (Joshi *et al*., 2011). The mitotic index is one of the parameters for the assessment of cytotoxicity of chemical agents. The cytotoxic agent can alter the mitotic index either by reducing or increasing the mitotic index, which is an indication of the mutagenicity of the agent (Grant, 1994). In the present study, the *A.cepa* chromosome assay was employed to investigate the mutagenic and antimutagenic potential of *A. terreus* extract.

4.2.2. Materials and Methods

The meristematic cells in the root tip of *A.cepa* were used for the assessment of mutagenicity and antimutagenicity of ethyl acetate extract of *A. terreus*. The mutagenicity and antimutagenicity of the extract were studied according to the protocol by Fiskesjo (1985) and Fernandes *et al*. (2007) with slight modifications.

4.2.2.1. Mutagenicity Assay

Mutagenicity assay was performed according to the method described by Fernandes *et al*. (2007). *A.cepa* roots were exposed to different concentrations i.e., 100, 50, 25, 12.5 and 6.25µg/ml of the ethyl acetate extract of *A. terreus* for 48 hours. Distilled water and cyclophosphamide were used as negative and positive controls respectively. The root tips were collected after 48 hours and the squashed roots stained with aceto-orcein were observed for normal and abnormal/aberrant dividing cells among a total of scored 2000 cells. The mitotic index was calculated as,

$$\text{Mitotic index percentage} = \frac{\text{No. of cells undergoing mitosis}}{\text{Total number of cells}} \times 100$$

$$\text{Percentage of chromosomal aberrations} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells}} \times 100$$

4.2.2.2. Antimutagenic Effect (Prajitha and Thoppil, 2016).

Pre-Treatment: The onion roots were treated with three different concentrations of the extract for 24 hours and then it was exposed to cyclophosphamide for 24 hours.

Post-Treatment: The onion roots were exposed to cyclophosphamide for 24 hours before it was transferred to different concentrations of the extract. After 24hours of incubation, the roots were collected and fixed for staining using aceto-orcein described by Fiskesjo (1985).
Percentage reduction of CP induced chromosomal aberrations (CA) was calculated using the formula:

RCA= (a-b)/ (a-c) X 100

RCA: percentage reduction of CP-induced chromosomal aberrations
a: percentage of CP induced CA; b: percentage of fungal extracts and CP-induced CA
c: percentage of distilled water-induced CA.

4.2.2.3. Statistical Analysis

Statistical analysis was carried out using Analysis of Variance (ANOVA), followed by Dunnett's multiple comparison tests using GraphPad Prism - version 5. The differences were significant when is $p < 0.05$.

4.2.3. Results

The mutagenic and antimutagenic potential of five different concentrations of the ethyl acetate extract (100, 50, 25, 12.5and 6.25µg/ml) was assessed by observing the change in mitotic index and mitotic aberration index in *A. cepa* treated with the test samples comparing with the negative control.

4.2.3.1. Mutagenicity Assay

The results of mutagenicity assay of *A. terreus* extract in *A. cepa* root cells are presented in figure (5) and (6). The roots treated with highest concentration of the extract i.e., 100 µg/ml showed lowest mitotic index (5.98%) where as the mitotic index of lowest concentration i.e. 6.25µg/ml treated roots (6.15%) were similar to the negative control values (6.31%) as shown in figure.(5).

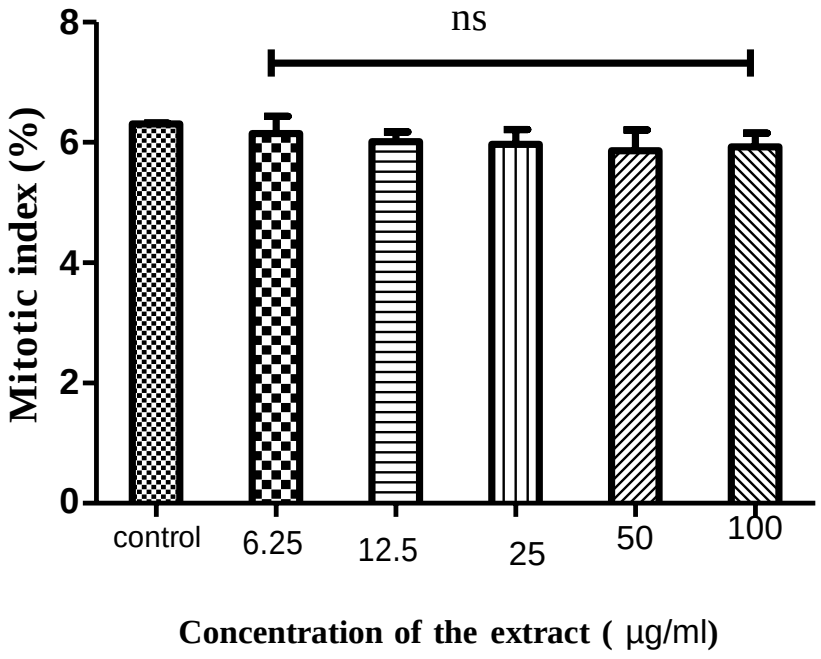


Fig (5): Effect of ATE on mitotic index of *A.cepa* root meristematic cells.

ATE- *A. terreus* extract;

ns: not significant (control vs. extract).

The mitotic aberration index of the lowest concentration (6.25µg/ml) treated roots was 0.136% which was similar to the natural or spontaneous chromosome abnormalities of the negative control which was found to be 0.13. The highest aberration index was found at a concentration of 50µg/ml.

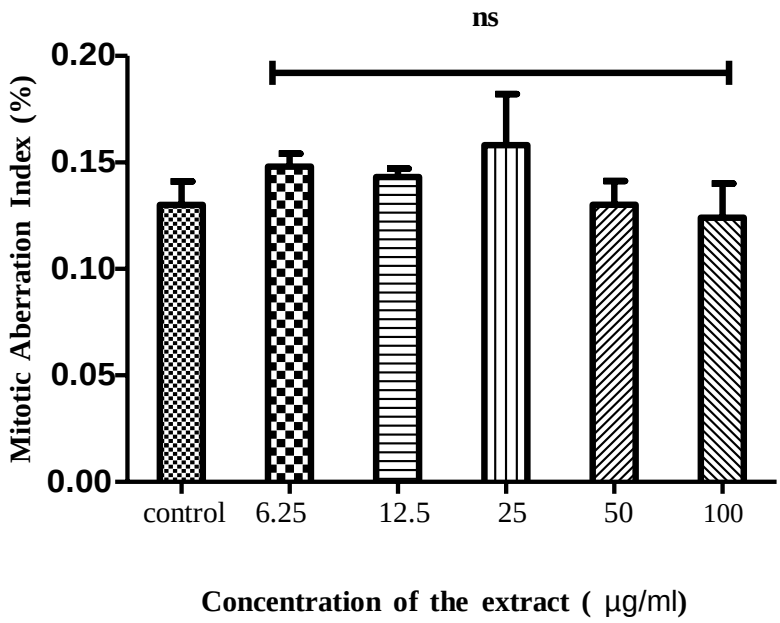


Fig (6): Effect of ATE on mitotic aberration index of *Allium cepa* root meristematic cells.
ATE- *A. terreus* extracts: ns:not significant (control vs. extract).

4.2.3.2. Antimutagenicity Assay

Pre-treatment

The results of the antimutagenicity assay by pre-treatment are presented in figure (7). The results showed that the extract exhibited a protective effect on the root cells against mutagen-induced toxicity. There was a significant reduction in cyclophosphamide-induced chromosomal aberrations at different concentrations ($p<0.05$). The percent reduction in DNA damage in the pre-treatment assay was found to be 0.09%, 12%, 23%, 37%, and 47%, for the concentrations 6.25, 12.5, 25, 50 and 100µg/ml respectively. In the pre-treatment protocol, it was observed that there was a concentration-response decrease in the chromosomal aberrations in *A. cepa* cells.

Post-treatment

The microscopic examination of the meristematic cells revealed that the roots exposed to cyclophosphamide showed inhibition of cell division at all treated concentrations of the extract. The cells were found to be in interphase and various morphological changes were observed in all the tested concentrations. The observed morphological changes in the *A.cepa* cells were

found to be complete disruption of cells, cytoplasm shrinkage and interphase cells which vary at different concentrations.

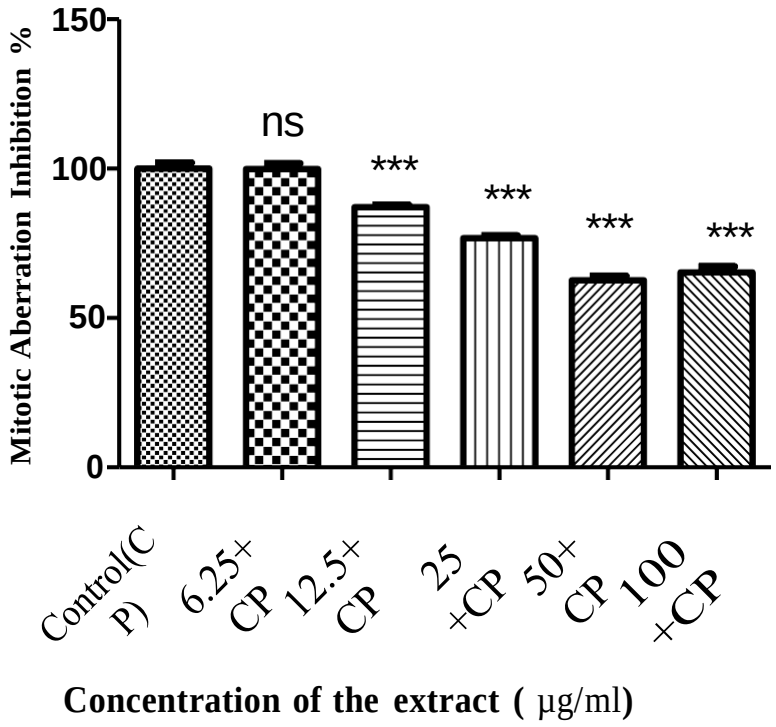


Fig (7): Effect of ATE on CP-induced mitotic aberration index in *A. cepa* root cells.
CP=cyclophosphamide; ns: not significant;*** p< 0.001
(Control vs. extract).

4.2.4. Discussion

The mean numbers of chromosomal aberrations found with the five concentrations of the extract were statistically similar to that of the control values indicating that the extract is not mutagenic to onion meristematic cells. Similar kind of results was reported in earlier studies (Malini *et al.,* 2010; Roberto *et al.,* 2016).

The results of the pre-treatment method indicated that in the antimutagenicity experiments, a combination of various concentrations of *A. terreus* extracts prior to exposure with the mutagenic agent cyclophosphamide exhibited a protective effect in *A. cepa* meristematic cells. *A. terreus* extract was effective in reducing DNA damage and is therefore considered chemo-preventive and antimutagenic. Similar studies were conducted by many researchers to demonstrate the mode of action of antimutagens of natural origin in *A. cepa* (Singh *et al.,* 2009; Prajitha and Thoppil, 2016; Akwu *et al*., 2019; Pereira *et al*., 2020). On the basis of the previous

studies and the results of the present work, it can be inferred that the biologically active compounds present in the extract favored the *A.cepa* cells by acting as a protective barrier that prevented the cyclophosphamide to interact with the DNA.

5. Study on the Growth Parameters and Antimicrobial Properties of *A. terreus*

In the previous chapters, it was confirmed that among all the three selected fungi, *A. terreus* exhibited significant antioxidant activity and a high presence of phenolics. Also, it was found that the crude ethyl acetate extract of *A. terreus* did not exhibit any mutagenicity in bothbacterial and plant models. On the other hand, the fungi showed significant antimutagenic capacity against cyclophosphamide and sodium azide. Hence, a further detailed study was conducted for *A. terreus* for its optimum growth parameters.

5.1. Optimization of Growth Medium and Physico-Chemical Parameters for High Yield Biomass Production of *Aspergillus terreus.*

5.1.1. Introduction

Optimal culture condition has a major role in the growth and fungal biomass accumulation, favoring the maximal secretion of metabolites. The product yield can be increased by optimization of process parameters viz., physical (temperature, salinity, pH, light, incubation time) and chemical factors (media components, carbon or nitrogen sources, trace elements) (Llorens *et al.*, 2004). The present study was carried out to evaluate various biochemical parameters to establish optimum growth conditions for the maximal production of bioactive secondary metabolites by *A. terreus.*

5.1.2. Materials and Methods

Optimization of the physico-chemical parameters for the production of bioactive secondary metabolites was performed in potato dextrose broth. The harvested thick mycelial mat was dried and the biomass yield was expressed as dry weight of mycelia (mg/25ml). The bioactive metabolite production was estimated in terms of DPPH radical scavenging activity (Mathan *et al.*, 2013).

5.1.2.1. Optimization of Media Parameters for Maximum Metabolite Production

In order to evaluate a suitable growth medium, *A. terreus* was grown in different culture media (Potato dextrose broth, Czapek's Dox broth, Sabouraud dextrose broth, Potato Carrot broth and Malt extract broth). The effect of carbon sources on metabolite production was assessed by incorporating different carbon sources such as dextrose, lactose, sucrose, fructose, maltose, starch, cellulose, and carboxy methyl cellulose into PDB. The effect of nitrogen source was determined by supplementing 1% of different nitrogen compounds such as peptone, yeast extract, malt extract, ammonium chloride, ammonium sulfate and urea to PDB. The mycelial weight and antioxidant activity were recorded at the end of the incubation period.

5.1.2.2. Optimization of Physicochemical Parameters for Maximum Metabolite Production

The fungus was allowed to grow under different temperatures (23°C, 28°C, 37°C, and 42°C) in order to optimize the standard temperature for growth. The effect of pH was evaluated by growing the fungi in PDB at different pH (4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5). Different concentrations of NaCl (3, 4, 5, 6 and 7%) were used to test the effect of salinity on mycelia growth and bioactive metabolite production. After 14 days of incubation, the mycelial dry weight and radical scavenging activity were determined.

5.1.2.3. Statistical Analysis

All experiments were carried out in triplicate using the aforesaid variables. The data were analyzed and expressed as mean ± standard deviation (SD). Statistical analysis was conducted using Analysis of Variance (ANOVA) for unpaired observations between the control and experimental samples, followed by Dunnett's multiple comparison tests using GraphPad Prism 5.

5.1.3. Results

5.1.3.1. Optimization of media parameters for maximum metabolite production

The extract derived from the fungus grown in PDA medium exhibited the highest antioxidant activity (84%; $p<0.05$). *A. terreus* showed a profuse growth and highest metabolite production in the PDA broth when compared with the other tested media. Moderate growth and DPPH scavenging activity was observed in potato carrot broth, malt extract broth, Sabouraud dextrose broth, and Czapek'sDox broth, respectively.

Among all the tested carbon sources, sucrose was found to be the best carbon source for antioxidant metabolite production ($p<0.001$), which was followed by cellulose and maltose. Biomass production was found to be high in the medium supplemented with cellulose followed by sucrose and dextrose. Carboxy methylcellulose was the least utilized compound by the fungus.

Maximal biomass production was noted in the culture medium supplemented with urea, followed by $(NH_4)_2SO_4$ and NH_4Cl. With reference to the DPPH scavenging activity, supplementation of $(NH_4)_2SO_4$ and NH_4Cl, gave the maximum results ($p<0.001$), followed by the malt extract ($p<0.01$).

5.1.3.2. Optimization of physical parameters for maximum metabolite production:

From the results, it was evident that the fungi exhibited growth at its maximal level and metabolite production in the acidic pH (pH 5.5). The maximal growth and DPPH activity was observed at 28^0C followed by 37^0C. There was a decrease in both biomass and antioxidant bioactive production at 23^0C and 42^0C. *A. terreus* showed a profuse growth and biomass production at salinity 4 %. The secondary metabolite production was higher at 7% NaCl.

5.1.4. Discussion

Among different nutrient media, the PDA medium was found to be the best one for high yield biomass and metabolite. The current observation is in parallel with the previous report (Goutam *et al*., 2016; Akilandeswari and Pradeep, 2017), where PDB and Sabouraud's dextrose were found to be better nutrient media for the growth and metabolite production of *A. terreus*. Mathan *et al*. (2013) and Bhattacharyya and Jha (2011) reported a profuse growth and maximal metabolite production by *Aspergillus* on all the carbon sources and sucrose-supplemented media.

According to the reports by Arora and Chandra (2010), on the assay of antioxidant potential of two *Aspergillus* isolates by different methods under various Physico-chemical conditions, higher activity was observed in sucrose as a carbon source followed by dextrose and maltose which are in consonance with the present study. From the results of the effect of nitrogen

sources, it was noted that there was no distinctive correlation between biomass production and antioxidant activity of the crude extract of the fungus. This indicates that the production of metabolites with potential antioxidant activity varies upon the supplemented nitrogen sources. The results on the influence of temperature (Bhattacharyya and Jha, 2011; Jain and Pundir, 2011) and salinity (Masuma *et al*., 2001) are in parallel with the earlier reports.

The findings of the present work suggest that the formulation of growth media and optimized physical and chemical parameters conditions will be helpful in the mass cultivation of the fungus for its industrial-scale production of bioactive molecules.

5.2. Evaluation of Antimicrobial Activity of *A. terreus* against Certain Pathogens

5.2.1. Introduction

The rapid developments in the field of cancer therapy over decades are always encountered by immune suppression and the abundance of risk factors associated with infection, which is one of the biggest problems faced by cancer patients seeking cancer treatment. Immune-compromised persons receiving chemotherapy and radiotherapy are also more vulnerable to secondary infections with pathogenic microorganisms (Panghal *et al*., 2011). In high-risk cancer patients, heavy antibiotic use can contribute to the development of drug resistance in microorganisms (Rolston *et al*., 2007). Fungi are possible sources of bioactive metabolites of pharmaceutical and industrial importance (Bala *et al*., 2011). Hence, researchers are turning their attention to the isolation and identification of novel antimicrobials from natural sources including fungal species. In this context, a study was focused on the evaluation of the antimicrobial potential of ethyl acetate extracts of *A. terreus*.

5.2.2. Materials and Methods

The crude ethyl acetate extract was evaluated for its antimicrobial activity by well diffusion assay **(**Magaldi *et al*., 2004**)** and Minimum Inhibitory Concentration was evaluated.

5.2.2.1. Well diffusion assay (Magaldi et al., 2004)

The test culture included Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeroginosa* (ATCC 27853), and Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883) and fungal species, *Candida albicans* (ATCC 10231). Streptomycin and Clotrimazole were used as a positive control for bacteria and fungi respectively. The percentage of growth inhibition was calculated as the ratio between growth inhibition by the extract and that of the standard antibiotic.

$$\text{Growth inhibition \%} = \frac{\text{Zone of growth inhibition by the extract}}{\text{of growth inhibition by standard antibiotic}} \times 100$$

5.2.2.2. Minimum Inhibitory Concentration (Varaldo, 2002)

The antibacterial activity of the fungal extract sample was tested against the bacterial isolates by micro-dilution method using a 96 well microtitre plate by measuring optical density at 540nm. After incubation for 24 hrs, the MIC was determined as the lowest concentration ofthe test agent, which resulted in the confirmed inhibition of the growth of tested microorganisms.

5.2.2.3. Statistical Analysis

The experiments were conducted in triplicates and the data were expressed as mean ± standard deviations (SD), analyzed by one way ANOVA followed by Bonferroni's Multiple Comparison Test.

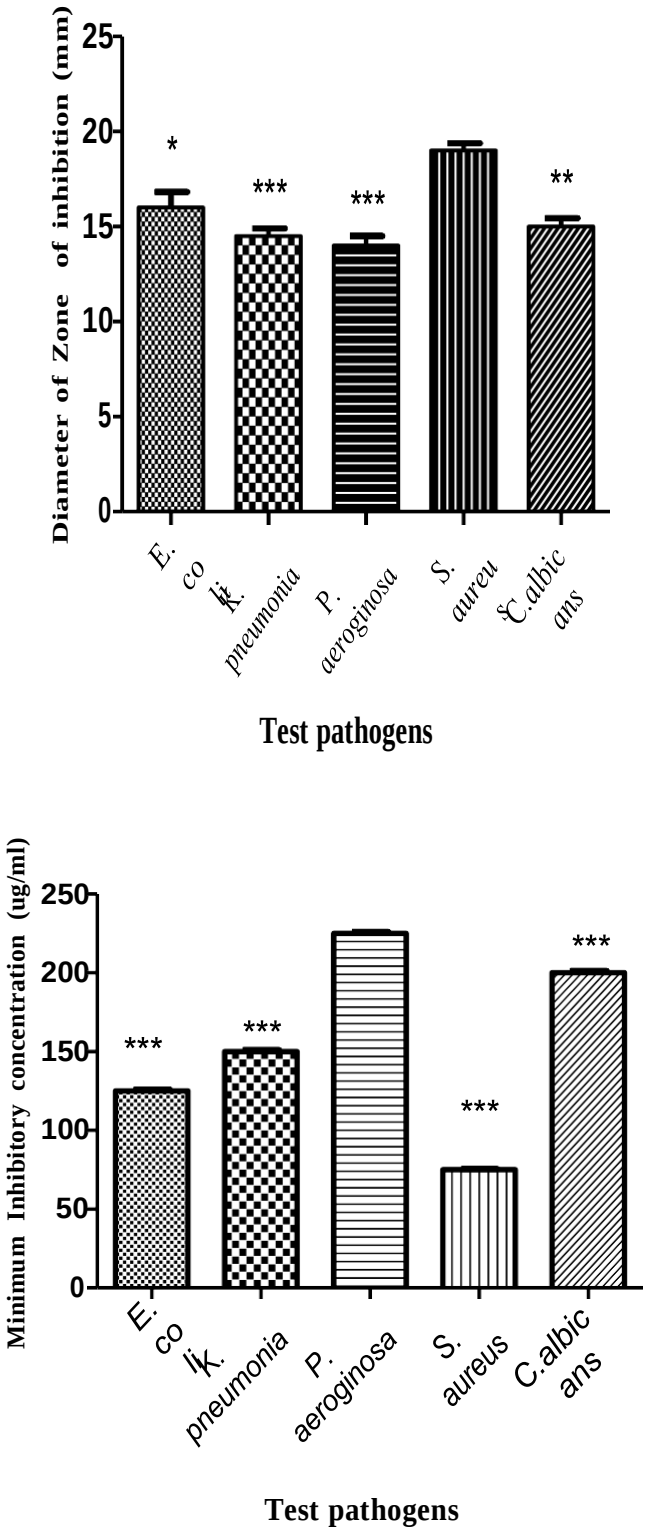
5.2.3. Results

5.2.3.1. Well Diffusion Assay

The result of antimicrobial activity by well diffusion assy is given in figure (8 a). At 1000 g/ml, *A. terreus* extract showed the highest zone of growth inhibition on *S. aureus* (19±0.67mm), followed by *E.coli* (16±1.41mm). The gram-positive bacteria, *P. aeroginosa,* were the least affected microbe by the antimicrobial compounds of the fungi. The extract showed the highest inhibition percentage of 65.6% against *S. aureus*, which was followed by 55% growth inhibition on *E. coli.*

5.2.3.2. Determination of Minimum Inhibitory Concentration

The inhibitory activity varied significantly against all the clinical isolates with MIC value ranged between 75-225 µg/ml as shown in figure (8 b). The MIC value of the ethyl acetate extract of *A. terreus* showed highest activity (75µg/ml) against *S. aureus* and lowest (225µg/ml) against *P. aeroginosa.* The extract showed second highest antimicrobial activity against *E coli.*



a b

Fig (8): Antimicrobial activity of ATE (100µg/ml) against clinical pathogens.

a: *Well diffusion;assay b: Minimum Inhibitory Concentration*; *p< 0.05, **p< 0.01;***p< 0.001.

(*S. aureus* vs. other test organisms).

5.2.4. Discussion

Results of the present study revealed that *A. terreus exhibited a substantial antibacterial and antifungal activity against certain pathogenic strains. It can be inferred that* there are certain secondary metabolites in the extract having the potency of growth inhibition. Prabavathy and Nachiyar (2012) reported that the crude extracts of *Aspergillus* species showed a strong inhibition zone against *E.coli*, *Klebsiella* sp., and *Pseudomonas* sp. which is in consonance with the present result. The findings of Choudhary *et al*. (2004) showed that ethyl acetate extract of *A. terreus* culture exhibited an inhibitory effect on animal and plant pathogenic fungi including *C. albicans*. The findings of the current study will facilitate further studies on the evaluation of toxicity, isolation, and purification of the antimicrobials, structural elucidation, and clinical trials of the compounds that open an alternate way in the development of chemotherapeutic agents against resistant organisms.

6. Evaluation of Anticancer Properties of *A. terreus in Vitro* and *In Vivo*

In vitro cytotoxicity study of *A. terreus* was carried out on L929 fibroblast cells and HeLa cell lines according to Mosmann (1983). The *in vivo* toxicity studies were conducted in the Zebrafish embryos.

6.1. Evaluation of Cytotoxic Potential and Anticancer Properties of *A. terreus In Vitro*

6.1.1. Introduction

Natural compounds are considered attractive candidates for the formulation of anticancer drugs (Evidente *et al*., 2014). 49% of the anticancer drugs are derived either from natural products or their derivatives (Bernardini *et al*., 2018). The diverse metabolites synthesized from fungi belong to different structural patterns making them a distinguishable resource of immune-modulating as well as anticancer agents (Moradali *et al*., 2007). The present study was aimed to evaluate the antioxidant, cytotoxic and anti-proliferative activities of the ethyl

acetate extract of *A. terreus*, an important source of natural metabolites of pharmaceutical importance.

6.1.2. Materials and Methods

6.1.2.1. Determination of Cytotoxicity

Cytotoxicity of the ethyl acetate extract of *A. terreus* was evaluated using Methyl Thiazolyl Tetrazolium (MTT) assay (Mosmann, 1983) on HeLa (Uterine cervix cancer cells) cell lines, and L929 fibroblast cells.

6.1.2.2. Assessment of Morphological Changes

HeLa cells were treated with IC_{50} concentration of the extract and incubated for 48 hrs. Cells were imaged using an axiocam attached to an inverted microscope (20X magnification). Morphological changes induced by the extract were recorded.

6.1.2.3. Cell Cycle Analysis

The influence of ethyl acetate extracts of *A. terreus* on the cell cycle of HeLa cells were evaluated using flow cytometry.

6.1.2.4. Comet Assay

In the present study, L929 (fibroblast) cells were used for assessing the DNA damage repairing ability of the extract induced by hydrogen peroxide. The slides were photographed using an Inverted epi-fluorescent microscope and the comets were scored using Tritek comet scoring software and correlated statistically.

6.1.3. Results

6.1.3.1. Determination of Cytotoxicity

The resuts of cytotoxicity of the ethyl acetate extract of *A. terreus* is given in fig (9). Results indicated that the ethyl acetate extract of *A. terreus* possessed significant cytotoxicity on HeLa cells in a concentration-dependent manner, with the highest toxicity of 85.05% at 200µg/ml concentration ($p<0.05$). The IC_{50} value of the extract was found to be 92.68 µg/ml. Results of cytotoxicity in fibroblast cells indicated that the ethyl acetate extract of *A. terreus* did not exhibit significant toxicity to L929 cells. However, the cell viability was reduced in a concentration-dependent manner, with the highest viability of 95.34% at 6.25µg/ml concentration. LC_{50} value of the extract was found to be 165.69µg/ml.

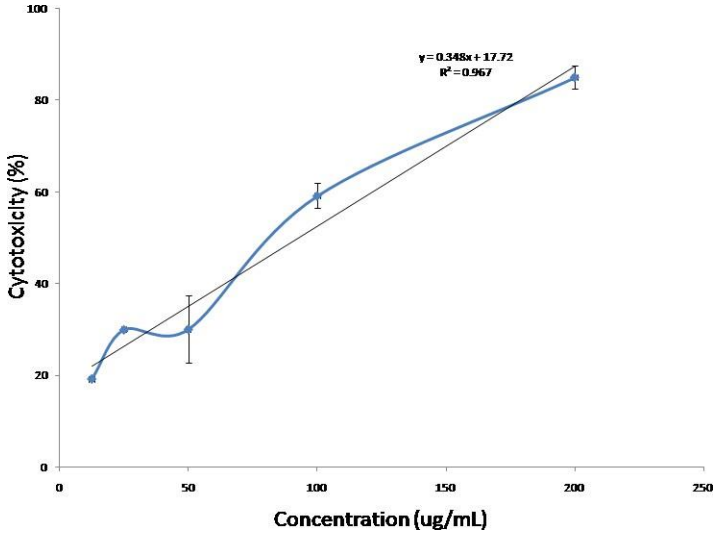


Fig **(9): Cytotoxicity of *A. terreus* ethyl acetate extract on HeLa cells determined using MTT assay**.

6.1.3.2. Assessment of Morphological Changes

Photomicrographs of HeLa cells treated with *A. terreus* extract showed that *the* extract induced certain morphological changes in the HeLa cells such as damaged cell membrane and cytoplasmic shrinkage. Ruptured cell membrane and cytoplasmic shrinkage are important features of necrotic and apoptotic cell death, respectively (Zhang *et al*., 2018).

6.1.3.3. Cell Cycle Analysis Using Flow Cytometry

A. terreus extract induced cell cycle arrest at the G2/M phase in HeLa cells. Control cells showed the regular distribution of DNA content across various phases of the cell cycle

whereas G0/G1 DNA content increased significantly in the extract-treated cells, suggesting the arresting of cell proliferation at the S and G2/M phases.

6.1.3.4. Comet Assay

The results showed that hydrogen peroxide treated cells (positive control) induced DNA damage in the cells. Cells treated with ethyl acetate extract of *A. terreus* were found to reduce the damage caused by hydrogen peroxide to a significant level as indicated by the tail length reduction.

6.1.4. Discussion

The observed cytotoxic activity of the extract against HeLa cells corroborates with previous studies where certain fungal extracts have been demonstrated to exhibit the activity against cancer cell lines (Artasasta *et al*., 2017; Koul and Singh, 2017; Danagoudar *et al*., 2018). The morphological changes observed in the extract-treated cells in comparison with the negative control confirmed the cytotoxic effect of the extract. Further detailed studies in this regard may give insight into the cytotoxic potential of the fungal extract induced either by necrosis or apoptosis. Jagetia and Venkatesha (2018) reported that *Aphanamixis polystachya* was capable of inducing DNA damage in HeLa cells, which was proved by employing the comet assay.

Thus, the present study has demonstrated the cytotoxic potential of *A. terreus* ethyl acetate extract on uterine cervix cancer cells. *A. terreus* can be considered a potential source of anticancer agents. Further studies are warranted in order to elucidate the molecular mechanisms of the extract-induced cytotoxic activity against the cancer cell line.

6.2. Evaluation of Anticancer Property of Ethyl Acetate Extract of *A. terreus* using *Danio rerio* (Zebrafish) as a model.

6.2.1. Introduction

The advancements of techniques in the field of cancer research explored various models from cancer cell lines to use genetically modified organisms for the in-depth evaluation of cancer development, progression and treatments (Xie *et al*., 2015). The newest model is the zebrafish, which has gained popularity due to its ease of handling in genetic and embryological experiments. Recently, zebrafish models have been used extensively to assess the toxicity of natural products or their derivatives (Thanh *et al*., 2016; Murugesu *et al*., 2019; Thiagarajan *et al*., 2019). Transparent embryos aid in assessing the effects of the test compound on the various developmental stages of organs in real time (Jayasinghe and Jayawardena, 2019).

In the present study, an evaluation of the developmental and genotoxic nature of ethyl acetate extract of the fungus, *A. terreus* was performed in zebrafish embryos. Also, an attempt was made to assess the molecular mechanism of the induction of apoptosis in zebrafish by targeting tumor suppressor genes.

6.2.2. Materials and Methods

The assessment of toxicity, anti-angiogenic and apoptotic properties of the extract was studied in wild and transgenic zebrafish (*Danio rerio)* at their embryonic developmental stage.

6.2.2.1. Determination of LC_{50}

The zebrafish embryo acute toxicity and mortality evaluation was conducted in accordance with OECD guideline 236 (OECD, 2013). TheLC_{50} was obtained by calculating the embryo mortality rate after 24 hours of exposure to various concentrations of the extract. LC_{50} was calculated using probit analysis, with the concentration at which all the embryos dead (LC_{100}) as the upper limit and the concentration at which all the embryos survived (LC_0) as the lower limit.

6.2.2.2. Evaluation of Developmental Toxicity (Panzica-Kelly *et al.,* 2010; OECD, 2013)

The developmental toxicity was evaluated for different concentrations (1-100ppm) of the extract. The embryos were observed for morphological changes for 5 days. The developmental stage at first exposure was the shield stage (10 hpf). Four types of toxicological assays, namely, developmental toxicity, cardiotoxicity, neurotoxicity, and hepatotoxicity were performed with observation and recording of lethal and sub-lethal endpoints. The effects on

development were analyzed on day 1 post fertilization followed by cardiotoxicity on day 2, neurotoxicity on day 3, and hepatotoxicity on day 5.

6.2.2.3. Assessment of Antiangiogenic Activity (Velez *et al*., 2012)

To investigate whether the extract can prevent vasculogenesis, transgenic zebrafish that expresses green fluorescent protein under the control of fli promoter (Tg [fli1: GFP]), the embryos were exposed to various concentrations of the crude extract. Embryos (n = 10) in triplicates were exposed to various concentrations of the compound at 10 hpf and were observed for the effects on inter-segmental Vessels (ISVs) and sub intestinal veins (SIVs) at 48 and 72 hpf. Phenylthiourea was added to prevent pigmentation. The embryos with exposure to DMSO served as a control group.

6.2.2.4. Evaluation of Apoptosis by Acridine Orange Staining

The effect of *Aspergillus terreus* extract on triggering apoptosis in Zebrafish embryos was studied using Acridine orange (AO) staining as explained by Tucker & Lardelli, (2007). To determine the extent of apoptotic cells per embryo, zebrafish embryos were treated with different concentrations of extract for 24 hours post-fertilization and stained with the AO dye in a concentration-dependent manner.

6.2.2.5. Gene Expression Profiling of p53, Bax and PUMA

The zebrafish embryos were exposed to different concentrations of the crude extract at 10hpf and RNA was extracted from the exposed and control embryos at 24 -hour post- fertilization (hpf), 48hpf and 72 hpf. The cDNA was synthesized and Gene expression of p53, Bax and PUMA were analysed. The experiment was performed in replicates of three, with thefindings analyzed using the t-test ($p < 0.05$). All calculations and tests were performed in Microsoft excel.

6.2.3. Results

6.2.3.1. LC_{50} Determination

The acute toxicity of ethyl acetate extract of *A. terreus* was assessed in zebrafish embryos by treating them with different concentrations of the extract for 24hpf and LC_{50} was found to be 104.47±0.29ppm.

6.2.3.2. Evaluation of Developmental Toxicity

The results of developmental toxicity of A. terreus extract is given in table(2) to (5).In all three treatments, the embryos remained alive until the fifth day of exposure. The embryo treated with higher concentrations of the extract showed deformations in the developmental stages from day 2. Delayed hatching, incomplete yolk sac consumption, defective swimming behavior, and a decrease in heart rate were observed at the higher concentrations beyond which the embryos were dead.

Table (2): Effect of ATE on developmental toxicity in *Danio rerio* on day 1 after exposure.

Developmental stages		Present				Absent			
		0.1ppm	0.2ppm	0.3ppm	0.4ppm	0.1ppm	0.2ppm	0.3ppm	0.4ppm
Lethal	Coagulation	-	-	-	-	✓	✓	✓	✓
	Non-formation of somites	-	-	-	-	✓	✓	✓	✓
	Non-formation of tail	-	-	-	-	✓	✓	✓	✓
Sub lethal teratogenic	Malformation of brain	-	-	-	-	✓	✓	✓	✓
	Malformation of otolith	-	-	-	-	✓	✓	✓	✓
	Malformation of eyes	-	-	-	✓	✓	✓	✓	-
	Malformation of tail	-	-	-	-	✓	✓	✓	✓
	Malformation of somites	-	-	-	-	✓	✓	✓	✓
	Malformation of notochord	-	-	-	-	✓	✓	✓	✓

	Malformation of yolk sac extension	-	-	-	✓	✓	✓	✓	-

Day 1: Developmental toxicity

Table (3): Effect of ATE on Cardiotoxicity in *Danio rerio* on day 2 after exposure.

Day 2: Cardiotoxicity

Developmental stages		Present				Absent			
		0.1ppm	0.2ppm	0.3ppm	0.4ppm	0.1ppm	0.2ppm	0.3ppm	0.4ppm
Lethal	Heart beat	✓	✓	✓	✓	-	-	-	-
Sub lethal Teratogenic	Malformation of heart	-	-	-	-	✓	✓	✓	✓
	Anaemia	-	-	-	✓	✓	✓	✓	-
	Defective cardiac functions	-	-	-	✓	✓	✓	✓	-
	Rhithemicity	✓	✓	✓	✓	-	-	-	-
	circulation	✓	✓	✓	-	-	-	-	✓

Table (4): Effect of ATE on Neurotoxicity in Danio rerio on day 3 after exposure.

Day 3: Neurotoxicity

Developmental stages		Present				Absent			
		0.1ppm	0.2ppm	0.3ppm	0.4ppm	0.1ppm	0.2ppm	0.3ppm	0.4ppm
Lethal	Heart beat/movement	✓	✓	✓	-	-	-	-	✓
Sub lethal Teratogenic	Delayed hatching	-	-	-	-	✓	✓	-	-
	Defective swimming behavior	-	-	✓	✓	✓	✓	-	-

Table (5): Effect of ATE on Hepatotoxicity in Danio rerio on day 5 after exposure.

Day 5: Hepatotoxicity

Developmental stages		Present				Absent			
		0.1ppm	0.2ppm	0.3ppm	0.4ppm	0.1ppm	0.2ppm	0.3ppm	0.4ppm
Sub lethal Teratogenic	Visual assessment of liver necrosis	-	-	-	dead	-	-	-	dead
	Malformation of gut associated organs	-	✓	✓	dead	✓	-	-	dead
	Incomplete yolk consumption	-	✓	✓	dead	✓	-	-	dead

6.2.3.3. Assessment of Anti-angiogenic Activity

The exposure of zebrafish embryos to the ethyl acetate extract of *A. terreus* resulted in disruption of the ISV formation leading to defective ISVs after 48hours post-fertilization at all the tested concentrations. A Steady decrease in ISV formation was observed with an increase in the sample concentration.

6.2.3.4. Evaluation of Apoptosis by Acridine Orange Staining

The apoptotic cells were observed as green fluorescent dots on a dark background. The number of green dots increased with increased concentrations of the extract when compared to the control. Hence, the results indicated that the ethyl acetate extract exhibits cell death by apoptosis mechanism in zebrafish embryos.

6.2.3.5. Gene Expression Profiling of p53, Bax and PUMA

From the expression profiling of the *p53* gene, it was observed that there was no significant change in gene expression in all three days implicating that the extract did not have any effect on the *p53* gene. *Bax* and *PUMA* gene expression is controlled by the expression ofp53. In this study, we observed that on the treatment of fungal extract on zebrafish embryos *Bax gene* remained down-regulated on day 1 of zebrafish development when compared with the control embryos and later showed an increase in expression in the upcoming days, while the *puma* gene expression in the treated embryos was down-regulated on the initial two days and later showed an increase on the third day. Though there was a change in the expression pattern of *bax* and *puma* in the fungal extract-treated embryos than in the control embryos, there was no change in the expression pattern of the *p53* gene. A conclusion that can be drawn from the present study is that the extract induces apoptosis by targeting other apoptotic genesas it did not have any direct influence on the *p53* gene.

6.2.4. Discussion

In the present study, the cytotoxicity and anticancer properties of *A. terreus* extracts were carried out in *Danio rerio* (zebrafish) embryos. The results showed that no developmental toxicity was observed in zebrafish embryos below 3ppm and the extract was able to induce apoptosis and reduce angiogenisis in the embryos. Abutaha *et al.* (2020) reported the toxicity of *Fusarium solani* in zebrafish Embryos. Alex *et al*. (2010), Jun *et al*. (2019), Duran *et al*. (2011) and Li *et al*. (2017) conducted studies on the development and mode of action of anti-angiogenic drugs derived from natural products and chemical sources, proving that angiogenesis is a limiting factor and that controlling the pathological processes associated with angiogenesis is one of the most important strategies in the development of therapeutics against tumor metastasis.

The combined in-vitro and in-vivo studies on the pro and anti angiogenic effects of natural compounds by many researchers (Beedie *et al*., 2016; Sheng et *al*., 2016; Huang *et al*., 2017; Chen *et al*., 2018) utilized zebrafish as an experimental animal, suggesting it to be a promising model vertebrate for high through put researches in drug discovery. More research is needed to determine the mechanism of action involved in the inhibition of angiogenesis and the anticancer effects of the fungus.

7. Molecular Docking Studies of *A. terreus* Metabolites

7.1. Introduction

Currently, chromatographic techniques are used to separate a single molecule from a mixture, while spectroscopy techniques are employed to elucidate the structural details of the compounds (Sasidharan *et al*., 2011). Computational biology and bioinformatics have the potential to transform not only the way pharmaceuticals are developed, but also to speed up the drug development process and reduce costs (Baskaran and Ramachandran, 2012). With molecular docking, the binding affinity between the drug and the target can be estimated *in silico*. As a result, *in silico* and *in vitro* screenings may be used to rapidly determine the toxicity of the drugs and compounds being examined, avoiding the necessity for further steps like *in vivo* and preclinical studies (Cava and Castiglioni, 2020). *In silico* docking studies were conducted with the identified compounds against HeLa cell proteins in order to determine the possibility of the components being drug candidates in cancer therapy.

In the previous chapters, it was reported that the *A. terreus* extract exhibited a significant cytotoxicity *in vitro* and *in vivo*. In the present study, bioactive compounds present in the extract were identified by spectroscopic techniques. Also, phylogenetic analysis was performed to identify the organism based on DNA sequencing. Molecular docking studies were conducted in order to assess the anticancer properties of the extracts against HeLa cell proteins.

7.2. Materials and Methods

7.2.1. Identification of the Compounds in the Extract by Spectroscopy

The identification of each metabolite present in the crude mixture of ethyl acetate extract was performed by FTIR and GC-MS analysis. IR spectrum was recorded in a spectrophotometer in the range 500– 4000 cm^{-1} at a resolution value of 4 cm^{-1}. The volatile compounds were identified on the basis of the NIST database by virtue of comparisons made of the actual mass spectral data acquired on each compound to the database in GC-MS analysis.

7.2.2. *In silico* Molecular Docking Studies

For the present study, four HeLa cell proteins were selected. The compounds showing a higher peak in the GC-MS analysis were selected as ligands for molecular docking with HeLa cell proteins. The structure of bioactive compounds was retrieved from the PubChem database. The LigPrep module was used to prepare the small molecules for molecular docking studies. The extra-precision (XP) mode of docking was used to generate the results.

7.3. Results

7.3.1. FTIR and GC-MS analysis

The FTIR Spectral data of the extract confirmed the presence of bioactive functional groups such as Alkynes (R-C≡C-H), alcohol/ phenol (–OH), and Aldehyde (C=O Stretch), Aromatic (C=C Bending), and Alkenyl (C=C Stretch). GC-MS analysis showed the presence of 14 molecules (table 6) and the fragmentation patterns of the mass spectra were compared with the data of known compounds stored in the National Institute of Standards and Technology (NIST) research library. The identification of the bioactive compounds is based on the peak area, molecular weight, and molecular formula.

Table (6): Major bioactive constituents identified in ethyl acetate extract of *A. terreus* by GC-MS analysis

Sl no	Retention time	Mol. weight	IUPAC Name	Chemical Formula

1	10.30	136	3-Cyclopenta[c]pyridazin-3-One,2,5,6,7-tetrahydro-	$C_7H_8N_2O$
2	10.62	220	Butylated hydroxyl toluene	$C_{15}H_{24}O$
3	17.41	154	TERREIN 1,2 Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl]-	$C_8H_{10}O_3$
4	20.94	240	Dodecyl acrylate	$C_{15}H_{28}O_2$
5	21.79	182	Benzoic acid,4-hydroxy-3 methoxy.,methyl ester	$C_9H_{1o}O_4$
6	22.71	196	7-tetradecene	$C_{14}H_{28}$
7	23.93	204	Aromadendrene	$C_{15}H_{24}$
8	25.8	278	Dibutyl pthalate	$C_{16}H_{22}O_4$
9	26.71	274	Propanoic acid,3-mercapto-,dodecyl ester	$C_1H_{30}O_2S$
10	28.85	210	3,11-Tetradecadien-1	$C_{14}H_{26}O$
11	31.51	308	1-Docosene	$C_{22}H_{44}$
12	33.29	314	5,8,11.Eicosatriynoic acid,methyl ester	$C_{21}H_{30}O_2$
13	33.56	436	9,12,15-Octadecatrienoic acid,2(acetyloxy)-1-(acetyloxy)methyl ester,[Z,Z,Z]	$C_{25}H_{40}O_6$
14	35.86	218	1-Propyl-3-(propen-1-yl)adamantane	$C_{16}H_{26}$

7.3.2. Molecular docking studies

The results of in silico docking studies of the ligand –protein interaction is given in the table (7). The analysis predicted that Caspase-3 protein showed highest interaction with (TERREIN 1,2 Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl]-) Also Nuclear factor NF-kappa-B p105 subunits - 3-Cyclopenta[c]pyridazin-3-One,2,5,6,7-tetrahydro-) interaction was high. Mucosal addressin cell adhesion molecule showed a better affinity with (Benzoic acid, 4-hydroxy-3 methoxy.,methyl ester).Cellular tumor antigen p53exhibited higher affinity towards -TERREIN 1,2 Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl].

Table (7): Docking score of bioactive compounds of A. Terreus extract with HeLa cell proteins.

Ligand	**(**Nuclear factor NF-kappa-B	(Caspase-3**)**	**(**Cellular tumor antigen p53**)**	**(**Mucosal addressin cell adhesion

	p105 subunits)			molecule 1**)**
Dibutyl phthalate	-5.801	-3.652	-1.85	-2.253
Benzoicacid,4 hydroxy-3 methoxy.,methyl ester	-7.975	-4.293	-2.475	**-3.52**
Benzoic acid,4-hydroxy-3 methoxy.,methyl ester	-3.808	-2.597	-0.942	0.18
7-tetradecene	-4.458	-2.751	0.64	-1.716
Butylated hydroxyl toluene	-4.995	-2.974	-0.484	-1.402
1-Docosene	-3.26	-1.388	-1.031	-1.563
Dodecyl acrylate	-3.674	-3.747	-0.825	-1.235
Propanoic acid,3-mercapto-,dodecyl ester	-3.797	-3.344	-2.326	-1.871
aromadendrene	-5.472	-2.297	-2.522	-1.544
3,11-Tetradecadien-1	-5.276	-3.01	-3.009	-2.44
3-Cyclopenta[c]pyridazin-3-One,2,5,6,7-tetrahydro-	**-8.179**	-3.572	-2.255	-1.923
9,12,15-Octadecatrienoic acid,2(acetyloxy)-1-(acetyloxy)methyl ester,[Z,Z,Z]	-3.472	-3.327	-0.49	-0.353
TERREIN(1,2 Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl]-	-6.988	**-5.429**	-1.85	-2.72
Terrain (1,2 Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl]-	-5.202	-3.725	**-2.475**	-1.063
1-Propyl-3-(propen-1-yl)adamantane	-3.547	-2.595	-0.942	-1.372
5,8,11.Eicosatriynoic acid,methyl ester	-2.384	-0.714	0.64	-1.506

7.4. Discussion

The spectroscopic analysis of the crude extract revealed the presence of biologically active compounds, which were found to exhibit binding interactions with the HeLa cell proteins. Among the identified compounds in the metabolites of *A. terreus*, terrain found to exhibit a good interaction with the caspase 3 proteins and Cellular tumor antigen p53, which indicates its anticancer property. Further *in vitro* and *in vivo* studies of the purified compounds could establish it as a candidate for drug development for cancer therapy.

Bungtongdee et al. (2019) used GC-MS to analyze the chemical composition of endophytic fungal isolates and found that bioactive constituents in the extract were responsible for the antimutagenic effects of the fungi. Similarly Munaeni *et al.* (2019) reported that the GC-MS analysis of the plant *Eleutherine bulbosa* extracts revealed various phytochemicals responsible for its antimicrobial potential. Hamed *et al.* (2018) identified the chemical agents responsible for the antimicrobial potential of *Aspergillus unguis* SPMD-EGY by employing spectroscopic analysis.

Elsayed *et al.* (2021) used *in vitro* and *in silico* analyses to determine the cytotoxicity and antibacterial capabilities of secondary metabolites isolated from *Tecoma stans*, which is similar to our observations Similar studies by Sahu *et al.* (2020); Krishnamoorthy and Balakrishnan (2014) and Ashwini *et al.* (2017) evaluated the antioxidant and anticancer potential of plants and fungi by employing *in silico* and *in vitro* methodologies, and suggested that the fungal metabolites can be considered as drug candidate for the cancer drug discovery process.

Summary and Conclusions

Natural products have received much interest in recent years for cancer prevention because of their numerous health benefits, the limitations of conventional cancer therapeutics with reference to toxicities. The survivability or life expectancy can be increased only by increasing the selectivity of anticancer agents or discovering novel compounds from natural resources. The purpose of the present research was to evaluate the anticancer properties of selected fungiboth in vitro and in vivo and the findings were summarized below.

- Among the three selected fungi namely *Aspergillus niger*, *Aspergillus terreus*, and *Penicillium citrinum*, the first one showed a significant antioxidant activity against free radicals which is one of the essential properties possessed by an anticancer compound.
- Analysis of the phytochemicals showed the presence of phenolics, saponins, reducing sugars, alkaloids, tannins, terpenoids and steroids.
- The *A. terreus* extract was found to be neutral to the prokaryotic and eukaryotic systems when tested for its genotoxicity.
- Ethyl acetate extract of *A. terreus* did not induce the frame shift and base-pair mutations as indicated by the *S.typhimurium* TA 98 and TA 100 strains respectively.
- The results of assays using *S. typhimurium* TA 100 and TA 98 strains indicated that the *A. terreus* extract exhibits a protective effect against sodium azide-induced mutagenicity.
- In the antimutagenicity assessment in the *Allium cepa* model, it was found that the fungal extract was capable of reducing the chromosomal aberrations induced by cyclophosphamide in the pre-treatment protocol. However, the results of the post-treatment protocol showed that the fungal extract could not lower the mutagen-induced toxicity.
- The anticancer activity of the ethyl acetate extract of *A. terreus* was assessed in cancer cell lines (HeLa and fibroblast) by employing various experiments *in vitro*. The extract demonstrated significant cytotoxicity in the HeLa cell line through apoptosis and cell cycle arrest. The findings emphasized the anticancer property of the crude extract *in vitro*.
- Studies of the extract on the zebrafish model indicated that the ethyl acetate extract of *A. terreus* possesses anti-angiogenic properties and induces apoptosis.
- The FTIR and GC-MS analysis proved the presence of biologically active compounds in the crude extract.
- *In silico* molecular docking studies suggested that some of the compounds (TERREIN; 1,2-Cyclopenten-1-one,4,5.hydroxy3-[1-propenyl]-;3-Cyclopenta[c]pyridazin-3-1,2,5,6,7- tetrahydro-; Benzoic acid, 4-hydroxy-3 methoxy methyl ester) present in the crude extract possess a strong binding affinity towards HeLa cell proteins (Caspase-3 protein, Nuclear factor NF-kappa-B p105, Mucosal addressin cell adhesion molecule Cellular tumor antigen, p53) , which may be responsible for its anticancer property.

- Owing to the potential antimutagenic and anticancer activities of the *A. terreus*, the fungus was subjected to mass cultivation to derive maximum secondary metabolites; optimum nutritional and physiochemical factors were determined.
- There is a good scope for further studies for isolating the active constituents from *A. Terreus* and using mammalian systems to characterize the active ingredients for their antimutagenic and anticancer activities.

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Publications

- Rajitha, P.B., & Naik, P. (2021). Optimization of growth medium and physicochemical parameters for high-yield biomass production and active metabolites from *Aspergillus terreus*. *Research Journal of Pharmacy and Technology*, *14*(6), 2924-2930.
- Rajitha, P.B., Prabhu, A. & Naik, P. (2022). *In vitro* anticancer activity studies of *Aspergillus terreus* on uterine cervix Cancer cells. *Research Journal of Chemistry and Environment,* 26 (4):180-186.

Presentations in Conferences

- Presented poster entitled 'Influence of culture media and different physical factors on growth and biomass production of *Aspergillus terreus'* in the National conference on Science and Technology-Reaching the Unreached-Recent Advances in physical, Chemical, Mathematical and Biological Sciences (8^{th} & 9^{th} September 217) at Mangalore University.

- Presented poster entitled 'Exploration of antibiotic activity of *Aspergillus terreus* –an in vitro study' In the National conference on Natural Bioactive Compounds and Potential Effects (27th January 2017) at St. Aloysius College (Autonomous), Mangaluru.

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PUBLICATIONS
AND
PRESENTATIONS

In vitro anticancer activity studies of *Aspergillus terreus* on uterine cervix Cancer cells

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Abstract

The exploration of new anticancer agents is in high demand owing to limitations of conventional therapies. This study was designed to evaluate the cytotoxic potential of Aspergillus terreus ethyl acetate extract on uterine cervix cancer cells. Secondary metabolites were extracted from broth cultures and total phenolic content was determined using the Folin-Ciocaltiu method. Antioxidant activity was evaluated using DPPH and ABTS free radical scavenging assays. The cytotoxic activity of the fungal extract was assessed using Methyl ThaizolylTetazolium bromide (MTT) assay on HeLa cells. Morphological changes in HeLa cells upon treatment with the extract were determined. The effect of the fungal extract on cell cycle arrest was evaluated using flow cytometry.

The extract showed a significant free radical scavenging activity. The total phenolic content was found to be 16.76 mg of gallic acid equivalents (GAE) per gram dry weight of the extract. The extract exhibited a cytotoxic effect on the HeLa cells with an IC_{50} value of 92.68 µg/mL. Cell cycle analysis revealed that A. terreus induced G0/G1 arrest confirming its anticancer activity. Results of the present investigation revealed that crude extract of the A. terreus possesses potential anticancer properties.

Keywords: *A. terreus*, Free radical scavenging, Phenolics, Cytotoxicity, Cell cycle analysis.

Introduction

Cancer is the second leading cause of mortality worldwide[11]. Global cancer predictions indicate that 27.5 million new cancer cases will be diagnosed each year by 2040[5]. Uterine cervix cancer ranks second among the cancers with a high mortality rate in women. It is diagnosed generally in the age group of 35-44 years. One of the causal factors for the initiation of cervical cancer is Human Papilloma Virus (HPV) infection that leads to the production of E6 and E7 proteins which in turn down-regulate the tumor suppressor genes such as p53 and pRb[30].

Commonly available treatment modalities for uterine cervix cancer include radical hysterectomy followed by radio- and chemotherapies depending upon the stages of metastasis. However, series of adverse effects are generally encountered with the standard chemotherapeutic regimen on the surrounding normal tissues which make cancer management a difficult task even today[25]. Therefore, worldwide so much of research is focusing on the exploration of novel anticancer agents with higher efficacy and minimal adverse effects. Natural compounds are considered as attractive candidates for the formulation of anticancer drugs[8]. 49% of the anticancer drugs are derived either from natural products or their derivatives[4].

The diverse metabolites synthesized from fungi belong to different structural patterns making them a distinguishable resource of immunomodulating as well as anticancer agents[23]. Filamentous fungi such as *Aspergillus*, *Penicillium and Talaromyces* are some of the most exploited sources for numerous bioactive compounds such as terpenes, statins and plant hormones possessing antifungal[8], anti-inflammatory[34] and anticancer agents[8].

Aspergillus terreus is a saprophytic fungus prevalent in warmer climates such as tropical and subtropical regions. It mainly grows in soil and has also been found in habitats such as decomposing vegetation and dust. The fungus has important industrial applications to produce organic acids such as itaconic acid and cis-aconitic acid and the enzyme xylanase[17]. Lovastatin (mevinolin), a secondary metabolite derived from *A. terreus* is used as a drug for hypercholesterolemia and reduces the risk of cardiovascular disease[9,13]. The fungus has been reported to produce other bioactive molecules such as terrain with melanogenesis inhibitor property[26] and cyclosporine A with an immune suppressant activity[1]. Several fungi are known to possess cytotoxic properties. Ethyl acetate extracts of fungi derived from the marine sponge *Neopetrosiachaliniformis* AR-01 have been reported with good cytotoxicity on WiDr colon cancer cells[3].

Danagoudar et al[7] reported the cytotoxic potential of endophytic fungi isolated from medicinal plant *Tragia involucrata* L. on MCF-7 and MOL-T4 cell lines. Several *Penicillium sp*. are reported as the producers of cytotoxic metabolites[16]. The present study was aimed to evaluate the antioxidant, cytotoxic and antiproliferative activities of the ethyl acetate extract of *A. terreus*, an important source of natural metabolites of pharmaceutical importance.

Material and Methods

Chemicals: Potato dextrose agar (PDA) was purchased from Hi-Media Limited and Merck, India. The solvents were purchased from Rankem, New Delhi, India. DPPH and ABTS were obtained from Sigma-Aldrich, USA.

Dulbecco's modified Eagle's media; FBS and antibiotic-antimycotic solution were procured from HiMedia, India. All the chemicals used were of analytical grade.

Fungi and culture conditions: *A. terreus* was procured from MTCC, Institute of Microbial Technology, Chandigarh, India, subcultured on potato dextrose agar (PDA) slants and preserved at 4°C. For the experimental purpose, the fungi were grown in a PDA plate by point inoculation, placing an agar disc at the center.

Extraction of secondary metabolites: *A.terreus* was grown on Potato dextrose Broth for fourteen days at room temperature. The secondary metabolite production was indicated by the change in broth color from pale yellow to dark brown. The broth surface was covered by a thick mycelial mat. At the end of the incubation period, the broth was filtered using Whatman No.1 filter paper and the mycelial mat was harvested. The thick mycelial mat was washed thrice with distilled water and allowed to dry at 50°C for 48 hours. The yield was expressed as the dry weight of mycelia (mg/25 mL).

The cell-free culture filtrate was mixed with an equal volume of ethyl acetate, three times in a separation funnel and vigorously shaken for 10 minutes. The separated organic layer was collected after allowing it to stand for 15 minutes. The solvent was removed by evaporation and the thickbrown colored semisolid extract was obtained.[22]

Antioxidant assays:

Determination of DPPH radical-scavenging activity:
Antioxidant potential of the secondary metabolites was assessed by DPPH-free radicals scavenging activity with slight modification.[36] The free radicals scavenging activity of the filtrate was analyzed by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH).3ml of the filtrate was mixed with 1 ml of a methanolic solution containing 0.1mM DPPH. The mixture was kept in dark for 30 min after homogenizing and the absorbance was measured at 517 nm against a blank and the percentage DPPH radical scavenging activity was calculated. Ascorbic acid was used as a reference compound. IC_{50} was calculated from the ascorbic acid concentration-dependent standard curve and was expressed in terms of mg/ml.

DPPH scavenging effect (%) = [(A0-A1/ A0) × 100]

A0 = absorbance of the control and A1= absorbance of the sample.

The assays were carried out in triplicate and the results are expressed as mean values.

ABTS Radical Cation Decolorization Assay: The antioxidant activity was assessed by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method proposed by Re et al.[27] ABTS was diluted in distilled water to 7 mM concentration. The stock solution was prepared by ABTS by mixing 7mM ABTS and 2.45 mM potassium persulfate to generate cations and incubating the mixture at room temperature for16 h in dark. The ABTS solution was diluted in methanol to obtain an absorbance of 0.70 at 734 nm. 10 µl of ethyl acetate extract (1000µg/ml) was mixed with 990 µl of ABTS solution and the absorbance was measured using a UV/visible spectrophotometer (Beckman Coulter, DU 730 Life Sciences) after 5 minutes ofincubation. Ascorbic acid (AA) was used as the positive control.

A dose-dependent curve was plotted by taking the concentration of the extract along the X-axis and absorbance along the Y-axis. The percentage of ABTS scavenging activity was calculated by the following formula:

ABTS·+ scavenging effect (%) = [(*A*0 − *A*1)/ *A*0] × 100

Total Phenolic Content: The total polyphenolics of ethyl acetate extracts of *A.terreus* were determined by the Folin-Ciocalteu method as described by Singleton et al[29]. 200 µl of ethyl acetate extracts (500 µg/µl) were mixed with 1.5 ml of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. To this solution, 1.5ml of 7.5%w/v aqueous Na_2CO_3 was added and vigorously mixed by vortexing.

This reaction mixture was incubated at 37 ºC in dark for 90 minutes. The absorbance was measured at 734 nm using a UV/visible spectrophotometer. The total phenolic content was determined from gallic acid equivalent standard curve using a concentration of 0-0.1 mg/ml.

Determination of cytotoxicity: HeLa Uterine cervix cancer cells were procured from National Centre for Cell Sciences (NCCS), Pune. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. They were sub-cultured upon attaining 70% confluence. Cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere and were used for the experiments after three consecutive passages.

Cytotoxicity of the ethyl acetate extract of *A. terreus* was evaluated using Methyl Thiazolyl Tetrazolium (MTT) assay.[24] HeLa cells were seeded onto 96 well microtiter plates at a seeding density of 5000 cells/well. After adherence, they were treated with different concentrations of the fungal extract viz. 12.5, 25.0, 50.0, 100.0 and 200 µg/mL for 48 hrs post-incubation with test compounds. MTT reagent (1 mg/ml) was added to the wells and incubated at 37°C for 4 hrs. Formazan crystals formed were solubilized using DMSO and absorbance was recorded at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). Percentage cytotoxicity of the extract was calculated with respect to untreated cell control. Using a concentration versus percentage cytotoxicity regression plot, the IC_{50} value was calculated.

Assessment of morphological changes: HeLa cells were seeded onto 6 well plates and incubated at 37°C and 5% CO_2 for 24 hrs. The cells were treated with IC_{50} concentration of the extract and incubated further for 48 hrs. Cells were imaged using an axiom attached to an inverted microscope (Primo Vert, Carl Zeiss) under 20X magnification. Morphological changes induced by the extract were recorded.

Cell cycle analysis using Flow cytometry: HeLa cells were seeded onto 6 well plates and incubated at 37°C and 5% CO_2 for 24 hrs. The cells were treated with IC_{50} concentration of the extract and incubated further for 48 hrs. Cells were harvested by removing the spent media, trypsinized, resuspended in fresh media and centrifuged at 1300 rpm for 5 minutes. Supernatants were discarded; cell pellets were re-suspended in 1X PBS and centrifuged at 1300 rpm for 5 minutes at 4°C. The washing process with PBS was repeated twice and the cell pellet was added with hypotonic lysis buffer containing 50 ug/ml RNase and 2 ug/ml PI.

The cells were incubated at room temperature in dark for 30 minutes and acquired for cell cycle using an analytical flow cytometer (Guava EasyCyte, Merck Millipore).5000 events were acquired on a red channel and DNA content in each phase of the cell cycle was recorded in the form of a histogram. Data were analyzed using InCyte software.

Results and Discussion

It has been advocated that despite the significance of fungi as an excellent source of pharmaceuticals owing to the production of structurally diverse metabolites, no fungi-derived agent has been approved as a cancer drug so far[18]. Thus, there is a good scope for evaluating of anticancer activity fungal species. In the present investigation, *A. terreus* has been evaluated for its cytotoxic potential against HeLa cell lines in conjunction with its antioxidant activity/constituents.

The crude ethyl acetate extract showed a significant DPPH scavenging activity (84%) against 0.1mM DPPH solution at 517nm. The IC_{50} value was found to be 38.72 µg/ml and that of the ascorbic acid was found to be 9.072 µg/ml (Figure 2A). Our results are in agreement with the previous findings reported by Yadav et al[35] and Saleh et al[28].

Antioxidants are the molecules that can alleviate or prevent damage from oxidation by reactive oxygen species; hence, they are able to safeguard the cells from many disorders associated with oxidative stress[20].

ABTS assay is an electron transfer-based assay wherein a dark blue colored, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$), is reduced by an antioxidant compound into colorless ABTS which can be measured spectrophotometrically. In the present study, the fungal extract showed 99.66 % scavenging activity at a concentration of 500µg/ml (Figure 2B). The presence of antioxidants in *A. terreus* extract that can donate hydrogen was determined by recording the decrease in the absorbance of the blue-green ABTS radical reaction. ABTS is an efficient method to analyze the antioxidant activity of both lipophilic and hydrophilic compounds[33]. From the results, it is evident that the ethyl acetate extract of *A. terreus*possesses potential antioxidant compounds as indicated by a concentration-dependent increase in the activity.

The total phenolic content determined by the Folin-Ciocalteu method was found to be 16.76 µg/mg extract (Figure 1). The results indicate that the tested extract is rich in polyphenols. Polyphenols are natural antioxidants, generally synthesized by plants and microbes as secondary metabolites.

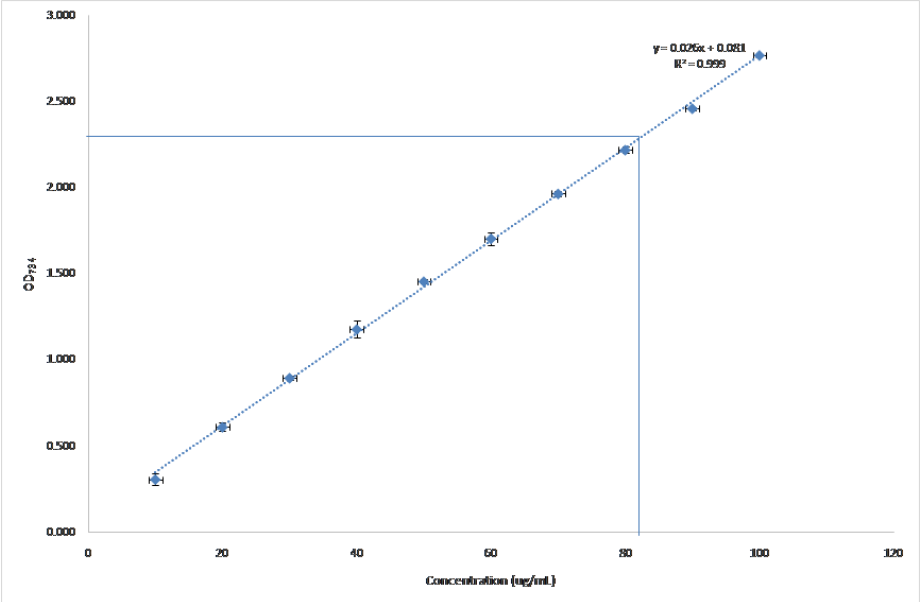
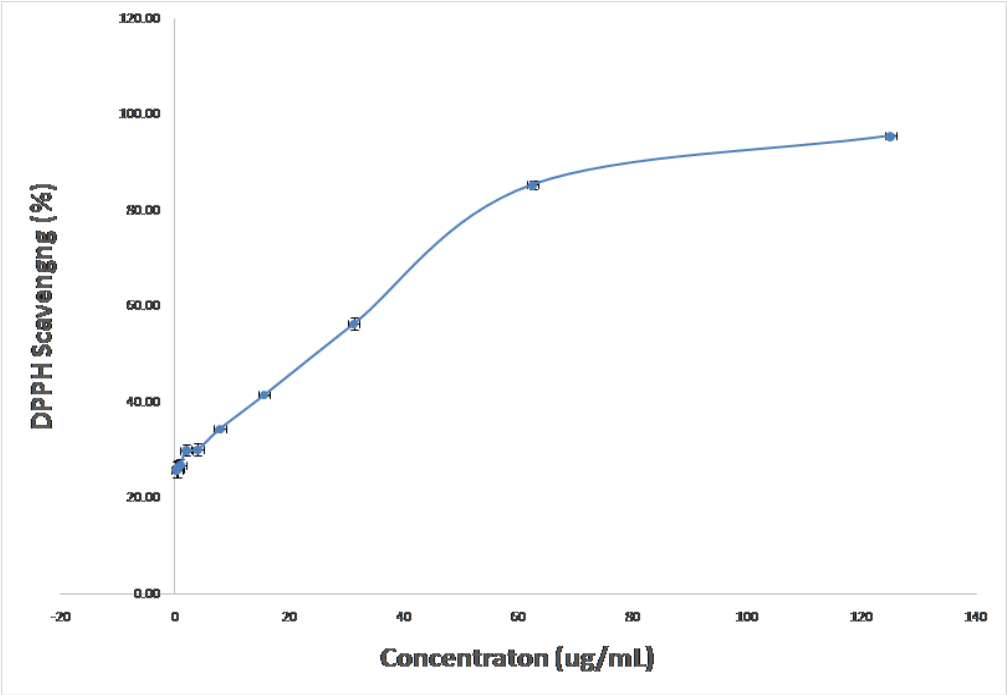


Figure 1: Total phenolic content of ethyl acetate extract of *A. terreus*.



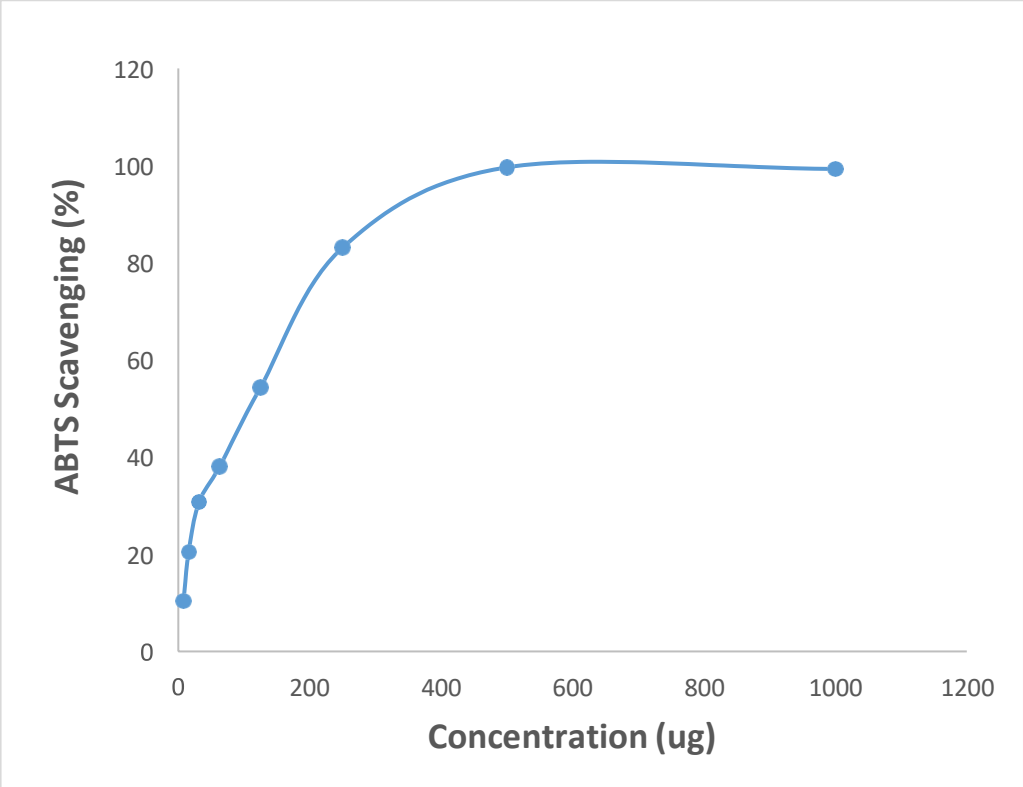


Figure 2 (A): DPPH free radical scavenging activity of *A. terreus* ethyl acetate extract at different concentrations. (B) ABTS radical scavenging activity of *A. terreus* ethyl acetate extract at different concentrations

It is known that polyphenolic compounds play an important role to resist health disorders including respiratory problems, cell injuries and neoplasm[31]. The current study is in parallel with an earlier report of Ibrahim et al[14], wherein ethyl acetate extracts of certain fungi exhibited higher equivalents of phenolics compared to other solvent extracts. The phenolic compounds present in the crude extract can be attributed to the radical scavenging property as evident from the two assays in the present study.

A similar kind of observation was also made in an earlier study demonstrating the positive correlation between the antioxidant potential of *Aspergillus* species and total phenolic content[2,35]. It is well-known that polyphenols with

potential antioxidant property not only act as chemopreventive but also as potential anticancer agents. Earlier studies have demonstrated the positive correlation between polyphenol with antioxidant property and anticancer activity[12,21].

Cytotoxicity of *A. terreus* extract on HeLa cells was assessed using an MTT assay. MTT assay is a colorimetric method which is based on the principle that MTT is utilized by a mitochondrial dehydrogenase present in the viable cells, succinate tetrazolium reductase into purple-colored formazan crystals whose absorbance is recorded at 570 nm[24]. Results indicated that the ethyl acetate extract of *A. terreus* possessed significant cytotoxicity in a concentration-dependent manner with the highest toxicity of 85.05% at 200 µg/ml concentration. The IC_{50} value of the extract was found to be 92.68 µg/ml. Percentage cytotoxicity induced by different concentrations of *A. terreus* extract is represented in figure 3.

The IC_{50} value of cisplatin taken as the reference standard against HeLa cell line was found to be 12.3µg/ml. The observed cytotoxic activity of the extract corroborates with previous studies where certain fungal extracts have been demonstrated to exhibit the activity against cancer cell lines[3,5,8].

Photomicrographs of HeLa cells treated with *A. terreus* extract are shown in figure 4. It can be seen that *A. terreus* extract induced certain morphological changes in the HeLa cells including damaged cell membrane and cytoplasmic shrinkage. Ruptured cell membrane and cytoplasmic shrinkage are important features of necrotic and apoptotic cell death respectively[37]. The observed fungal-extract morphological changes in comparison with the negative control confirmed the cytotoxic effect of the extract. Further detailed studies in this regard may give insight into the cytotoxic potential of the fungal extract induced either by necrosis or apoptosis.

A. terreus extract induced cell cycle arrest at G2/M phase in HeLa cells. Histograms of untreated control cells and cells treated with IC_{50} concentration of *A. terreus* extract are represented in figures 5A and 5B respectively. Control cells showed the regular distribution of DNA content across various phases of the cell cycle whereas G0/G1 DNA content increased significantly in the extract-treated cells suggesting the arrest at S and G2/M phases. There are reports on the production of antitumor secondary metabolites from several endophytic fungi by interfering with the regulation of the cell cycle[10,15,19,32].

Conclusion

Thus, the present study evaluated the antioxidant and cytotoxic potential of *A. terreus* ethyl acetate extract on uterine cervix cancer cells. *A. terreus* can be considered as a potential source of anticancer agents.

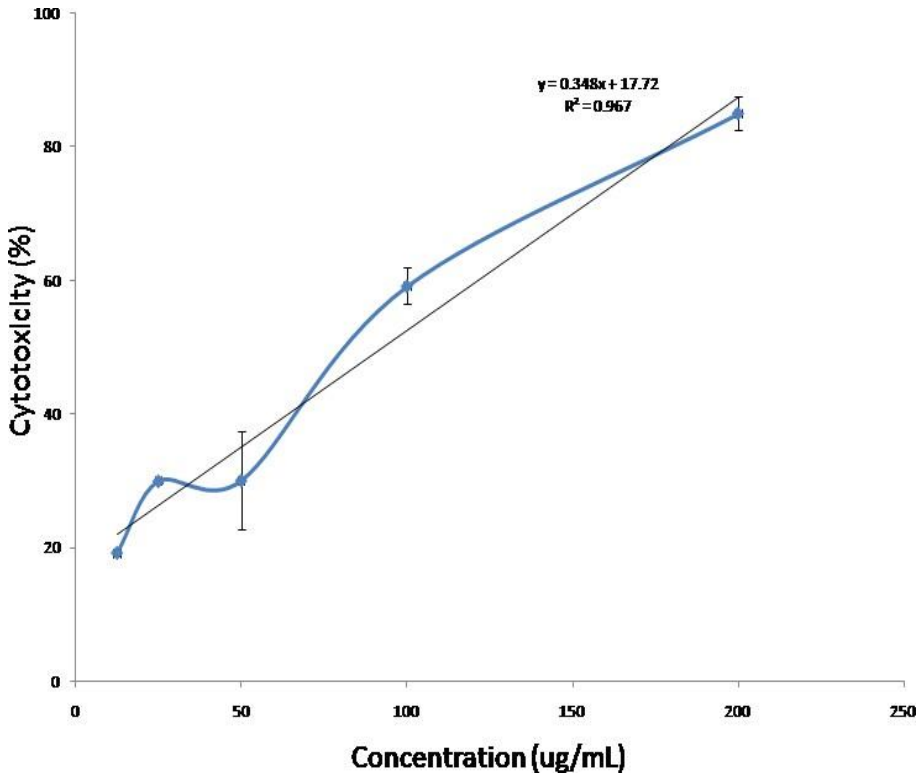
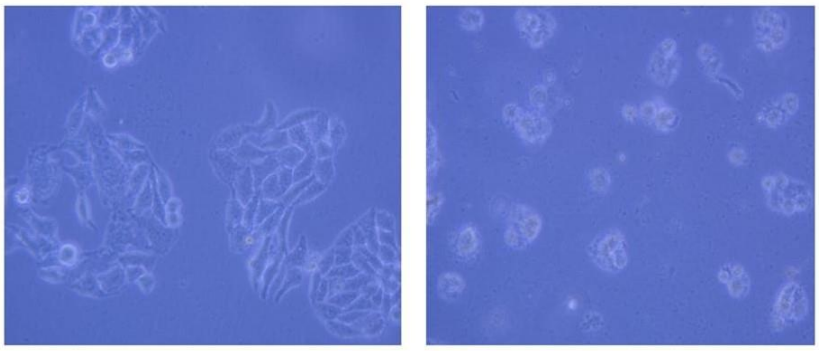
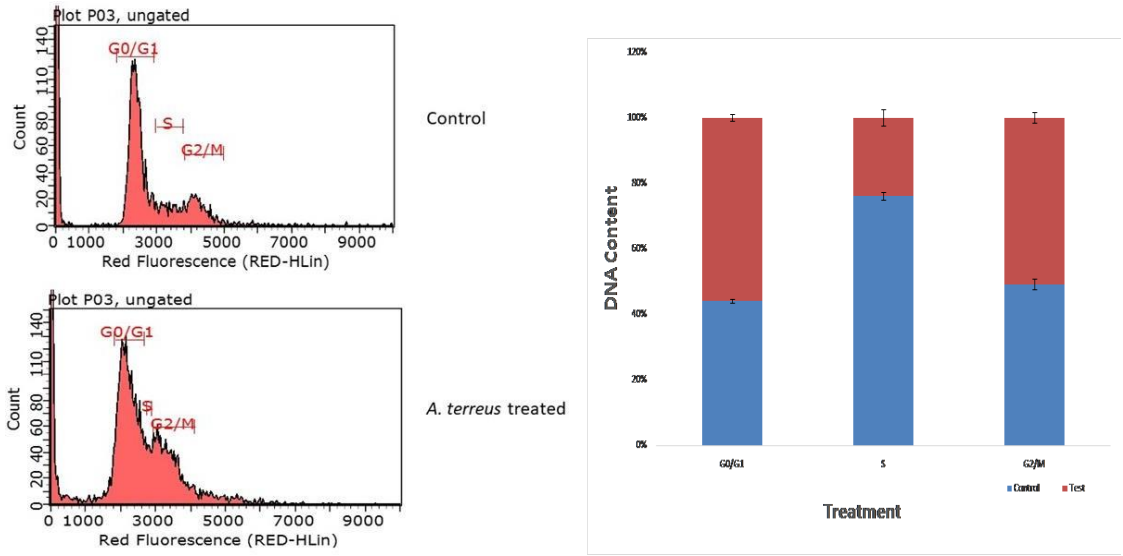


Figure 3: Cytotoxicity of *A. terreus* ethyl acetate extract on HeLa cells as determined using MTT assay. Values are expressed as mean ± SD (n = 3).



Control ***A. terreus treated***

Figure 4: Photomicrographs of HeLa cells treated with IC50 concentration of *A. terreus* extract. Images were captured using axiocam attached to an inverted microscope (PrimoVert, Carl Zeiss) and the software used was Zenlite Version 2012 at magnification 20X.



(A) **(B)**

Figure 5: (A) Histograms representing various phases of the cell cycle in HeLa cells treated with IC50 concentration of *A. terreus* extract. (B) Column diagram indicating the changes in DNA content in G0/G1, S and G2/M phases of the cell cycle of HeLa cells exposed to *A. terreus* extract. Values are expressed as Mean % ± SD.

Further studies are warranted in order to elucidate the molecular mechanisms of the extract-induced cytotoxic activity against the cancel line.

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(Received 09th October 2020, revised 02nd December 2020 accepted 20th January 2021)

ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Optimization of Growth Medium and Physicochemical Parameters for High-Yield Biomass Production and Active Metabolites from *Aspergillus terreus*

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ABSTRACT:
The selection of a nutrient medium and optimization of physicochemical parameters play a crucial role in the growth enhancement and production of bioactive secondary metabolites of fungi. The current study was to evaluate the influence of cultural conditions and physicochemical parameters affecting the growth and biomass production of *Aspergillus terreus.* Nutrient medium ((Potato dextrose broth, Czapek's Dox broth, Sabouraud dextrose broth, Potato Carrot broth and malt extract broth) in conjunction with physicochemical parameters such as temperature, pH, nitrogen sources, carbon sources, and salinity were assessed for optimal biomass production.The growth rate of the fungus was measured by determining the mycelial dry weight, and enhancement of antioxidant activity through DPPH scavenging potency in the culture broth. The highest growth rate of the fungus was observed in potato dextrose broth (PDB) compared to the other nutrient media at a significant level ($p< 0.05$). The present study demonstrated that PDB supplemented with cellulose and urea as carbon and nitrogen sources with a pH of 5.5 and 4% salinity at 28°C are the suitable conditions for maximal growth and biomass production. However, the antioxidants activity was at its peak level in PDA enriched with sucrose and ammonium sulphate at pH 5.5, and 7% salinity at 28°C. Thus, culture conditions, including nutrient components and physicochemical parameters play an important role in the growth rate *A. terreus,* to exploit the maximum yield for various biomedical applications.

KEYWORDS: Mycelial dry weight; DPPH assay*; Aspergillus terreus;* bioactive molecules; antioxidant activity.

INTRODUCTION:
Microorganisms are rich sources of bioactive compounds such as antibiotics, industrially enzymes, antioxidants, and other pharmacologically important molecules. Numerous studies indicate that, among all the microbes, fungi and actinomycetes have been found as the intense producers of a vast variety of bioactive metabolites[1].

These molecules are commonly referred to as secondary metabolites which vary structurally and functionally, and they range from intra- and extra-cellular low molecular weight compounds, proteins, polysaccharides or polysaccharide-protein complexes[2].Because of their diversity in nutritional and biochemical features, the production of metabolites varies with a slight change in their surrounding environment in terms of both quality and quantity[3]. Antioxidant compounds from various fungi have contributed immensely to the discovery of novel and potent drugs[4].

Aspergillus terreus is a common fungus grows in soil and plants. It is brownish in color and gets darker as it ages on culture media. Secondary metabolic machinery of *Aspergillus* species is a rich source of novel drug candidates and other industrially relevant enzymes and biomolecules[5]. The fungus is known for its production of

Received on 01.04.2020 Modified on 10.08.2020
Accepted on 08.10.2020 © RJPT All right reserved
Research J. Pharm. and Tech. 2021; 14(6):2924-2930.
DOI: 10.52711/0974-360X.2021.00513

a secondary metabolite, lovastatin, which is used as a drug for lowering blood cholesterol levels. Earlier studies indicated that *A. terreus* is a rapid producer of many bioactive molecules such as terpenes[6], statins[7], antifungals[8], anticancer agents[7] and polyketides[9]. Biologically relevant compounds have been isolated from the fungus, including sulochrin, terretonin, asterriquinone and butyrolactone terreineol[7], terreulactone A[10], terrain[11], terreic acid[12] and aspulvinones[13]. *A. terreus* is a well-known industrially explored fungus for the production of bioactive secondary metabolites[14]. Large scale industrial production of these compounds requires an extensive evaluation of nutritional and environmental parameters for the optimum growth of *A. terreus* in the culture lab[15]. However, selection of nutrient medium and providing physicochemical conditions depend upon the type of metabolite to be exploited. Both submerged fermentation (SmF) and solid substrate fermentation (SSF) are widely employed for large scale production of antioxidant metabolites[16]. Traditional optimization procedures of media components involve supplementation of inexpensively available different solid substrates with various C/N sources[17]. Industrial scale productions of enzymes from various *Aspergullus* spp. are carried out using inexpensive agro-bio wastes. Agro-bio wastes such as sugarcane bagasse[18], rice husk and rice bran[19], wheat bran[20], potato skin[21], sweet potato[22] and groundnut oil cake[23] have been utilized for the production of various carbohydrate hydrolyzing and proteolytic enzymes from the fungal species.

Optimal culture condition has a major role in the growth and fungal biomass accumulation, favoring the maximal secretion of metabolites[24]. The product yield can be increased by optimization of process parameters viz., physical (temperature, salinity, pH and light, incubation time) and chemical factors (media components, carbon or nitrogen sources, trace elements)[25]. It can be achieved either by employing statistical approaches like Plackett-Burman design[26], Response Surface Methodology[27,28], Central Composite Design[29] or by traditional methods. The present study was carried out to evaluate various environmental and biochemical parameters to establish optimum growth conditions for the maximal production of bioactive secondary metabolites by *A. terreus*.

MATERIAL AND METHODS:

Screening of selected microorganism:

A. terreus was procured from MTCC, Institute of Microbial Technology; Chandigarh, India. The culture was revived on potato dextrose agar (PDA) slants and stored at 4°C. Media components such as PDA, dextrose, galactose, sucrose, lactose, maltose, fructose, peptone, yeast extract, malt extract, urea, ammonium chloride, ammonium sulphate, were purchased from Hi-Media, India and Merck, India. Ethanol and ethyl acetate were purchased from Rankem, New Delhi, India. All the chemicals used were of analytical grade.

A. terreus was grown for fourteen days at room temperature. The secondary metabolite production was indicated by the change in broth color from pale yellow to dark brown. The broth surface was covered by the thick mycelial mat. At the end of the incubation period, the broth was filtered and the mycelial mat was harvested. The thick mycelial mat was washed thricewith distilled water and allowed to dry at 50°C in a hot air oven for 48 hours, and the yield was expressed as dry weight of mycelia (mg/25ml)[30].

DPPH radical scavenging assay:

The free radicals scavenging activity of the filtrate was analyzed by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH). 3ml of the filtrate was mixed with 1 ml of a methanolic solution containing 0.1mM DPPH solution. The mixture was kept in dark for 30 min after homogenizing and the absorbance was measured at 517 nm against the blank and the percentage DPPH radical scavenging activity was calculated. Ascorbic acid was used as a reference standard.

DPPH scavenging effect (%) = [(A0-A1/ A0) × 100]

A0 = absorbance of the control,
A1= absorbance of the sample.

Optimization of growth and bioactive metabolite production:

Optimization of the physicochemical parameters for the production of bioactive secondary metabolites was performed in potato dextrose broth.

Effect of different culture media:

In order to evaluate a suitable growth medium, *A. terreus* was grown in different culture media such as Potato dextrose broth, Czapek's Dox broth, Sabouraud dextrose broth, Potato Carrot broth and malt extract broth. The medium which showed maximum mycelial biomass accumulation and bioactive metabolite (antioxidant) production in terms of DPPH activity was used as the optimized medium for further study.

Effect of salinity:

The effect of salinity on mycelia growth and bioactive metabolite production was carried out in potato dextrose broth with different concentrations of NaCl (3%-7%). *A. terreus* was grown in PDB at room temperature for 14 days. The biomass and bioactive metabolite production were determined by quantifying the dry weight and DPPH assay, respectively.

Effect of pH:
25ml of potato dextrose broth with varying pH levels (pH 4.5-7.5) were inoculated with actively growing 5mm mycelial discs of seven days old fungal culture under aseptic condition and incubated at optimal physical parameters. After 14 days of incubation, thebroth was filtered using Whatman No.1 filter paper and mycelial dry weight and radical scavenging activity was recorded.

Effect of temperature:
The fungus was allowed to grow under different temperatures ranging from 23°C to 42°C keeping allother parameters constant. After 14 days of incubation, the mycelial dry weight and radical scavenging activity were determined.

Effect of carbon sources:
Different carbon sources such as dextrose, lactose, sucrose, fructose, maltose, starch, cellulose, carboxy methyl cellulose were used to assess their influence on the growth of *A. terreus* and metabolite production. 25ml basal medium was supplemented with 1% of each carbon source. The flasks were inoculated with the single mycelial disc (5mm) and incubated keeping optimal growth parameters constant. After incubation period mycelia weight and radical scavenging activity were determined.

RESULTS AND DISCUSSION:

Effect of nitrogen sources:
To study the effect of nitrogen source, 1% of different nitrogen sources such as peptone, yeast extract, malt extract, ammonium chloride, ammonium sulphate and urea were supplemented to 25ml of basal PDB medium individually and inoculated with 5mm mycelial disks of seven days old fungal culture under aseptic condition and incubated for 14 days. The mycelial weight and antioxidant activity were recorded at the end of the incubation period.

Effect of combined optimized conditions:
The effect of all the optimized conditions was also studied and determined biomass yield in conjunction with antioxidant activity. The PDB was supplemented with sucrose and ammonium sulfate as carbon and nitrogen sources respectively and the fungus was grown at a temperature of 28°C; pH 5.5 with 7% NaCl. At the end of incubation, the filtrate was checked for DPPH activity and dry weight of mycelia.

Statistical analysis:
All experiments were carried out in triplicate using the aforesaid variables. The data were analyzed and expressed as mean±standard deviation (SD). Statistical analysis was conducted using Analysis of Variance (ANOVA) for unpaired observations between the control and experimental samples, followed by Dunnett's multiple comparison tests using GraphPad Prism - version 5.

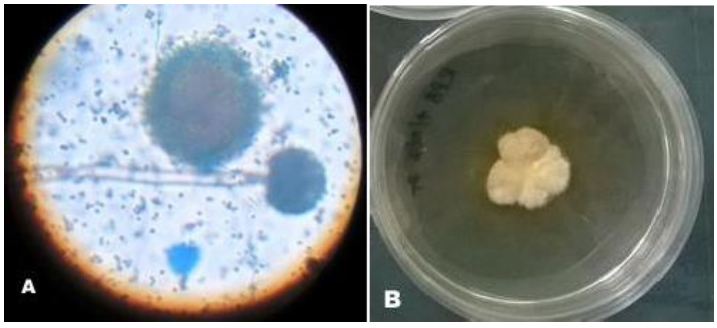

Fig.1: (A) Microscopic view of *A.terreus* under 40X magnification; B) Agar plug method

Fig (2): Submerged Fermentation of *A. terreus* in PDB

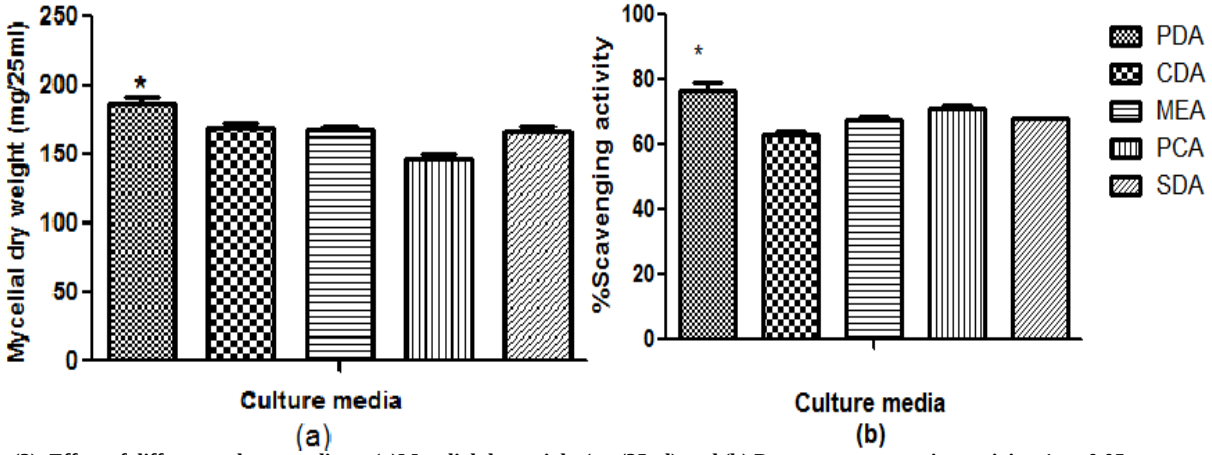

Fig (3): Effect of different culture media on (a)Mycelial dry weight (mg/25ml) and (b) Percentage scavenging activity; *p < 0.05 PDA-Potato dextrose agar, SDA- Sabouraud's dextrose agar, CDA-Czepek Dox agar, MEA-Malt extract agar, PCA- Potato Carrot agar

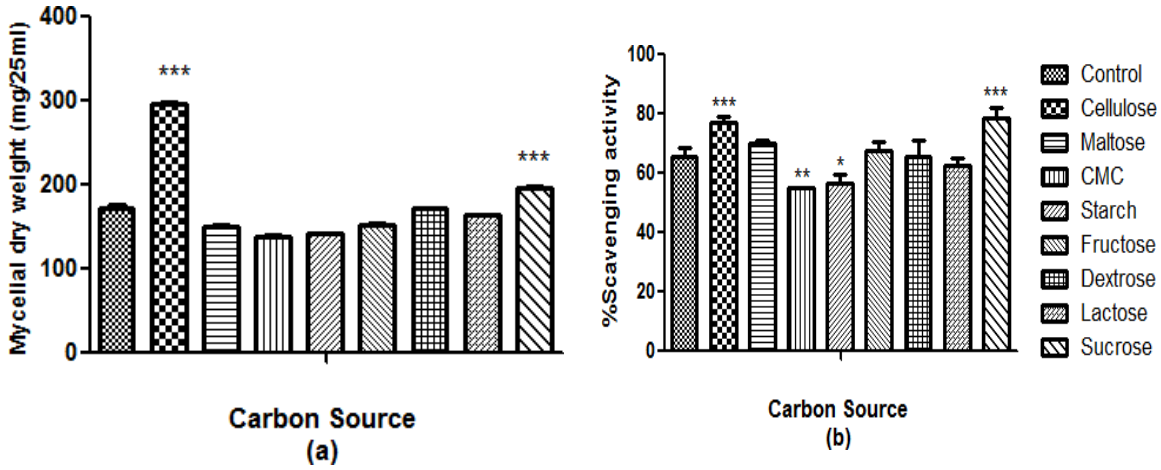

Fig (4): Effect of carbon sources on (a) Mycelial dry weight (mg/25ml) and (b) Percentage scavenging activity
***p<0.001; **p<0.01;*p<0.05

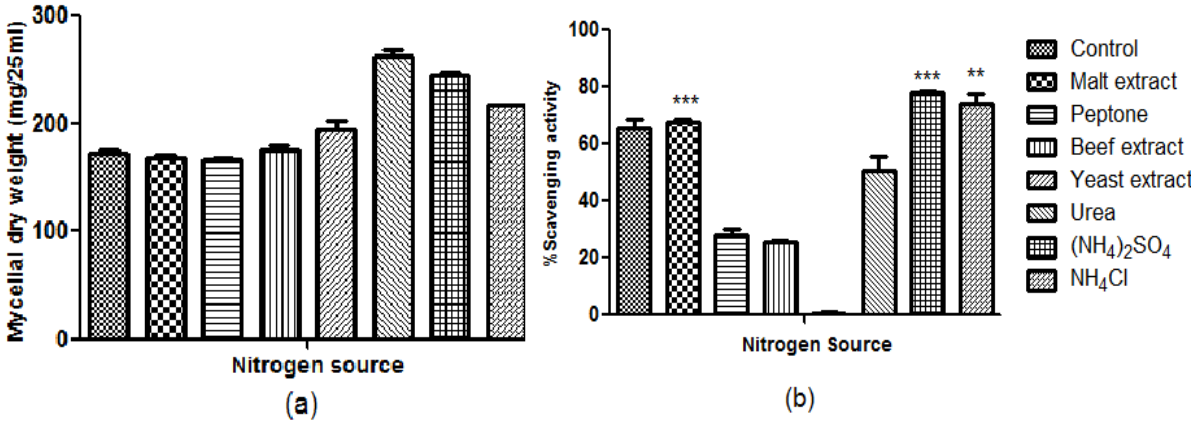

Fig (5): Effect of nitrogen sources on (a)Mycelial dry weight (mg/25ml) (b) Percentage scavenging activity;
*p< 0.05; **p<0.01; ***p<0.001

Table (1): Effect of pH, temperature and salinity on mycelial dry weight and Percentage scavenging activity

Parameters		Mycelial dry weight (mg/25ml)	% Scavenging activity
pH	Control (5.4)	170±1.41	65.45±3.03
	4.5	160 ±2.82	78.03±0.39***
	5.0	164±1.41	79.02±1.78***
	5.5	171±1.41***	82.47±0.31***
	6.0	167±2.83*	67.90±0.31
	6.5	156±1.41	69.28±3.79
	7.0	155±0.71	68.50±4.11
	7.5	152±4.24	73.49±0.16**
Temperature	Control (25)	173±2.83	62.97±1.55
	23	169±8.48	53.19±3.78
	28	267.5±4.94***	68.49±0.89*
	37	189±2.83*	63.31±0.66
	42	137.5±2.12	56.21±0.73
Salinity	Control (1)	173±2.83	65.45±0.70
	3	167±1.41	60.08±0.23
	4	389±2.83	63.30±0.93
	5	321±1.41	70.10±0.62**
	6	361±1.41	70.31±0.15**
	7	147±2.83	70.47±3.02**
Combination of the optimized parameters (28°C; pH 5.5 with 7% NaCl)	Control	173±1.41	65.32±2.03
	Modified media	253.6±3.92***	78.5202±1.78**

*p< 0.05; **p<0.01; ***p<0.001

Nutrient media and physicochemical parameters for growth of fungal species play a significant role in deriving the large-scale production of biomass and high yield of active metabolites[31,32]. In this context, the present study was conducted to optimize the physicochemical parameters for a high yield biomass production of *A. terreus* and its antioxidant potency.

Morphological identification and microscopic evaluation of the selected fungal cultures is the first and foremost requirement which helps to assess viability and growth patterns of the fungi. The colonies were observed to be typically leathery and cinnamon-buff to sand brown in color with brownish exudates and dark brownish on the reverse side. The colony characteristics and microscopic features of *A. terreus* on PDA medium were shown in Fig. 1. The secondary metabolite production was indicted by a change in the broth color from yellow to cinnamon brown as shown in Fig. 2.

In the present study, the crude extract of *A. terreus* cultured in different growth media showed a substantial antioxidant property in terms of DPPH free radical scavenging activity (Fig. 3b). Comparatively, the extract derived from the fungus grown in PDA mediumexhibited the highest antioxidant activity ((84%; p<0.05). The DPPH free radical scavenging activity of different fractions of *A. terreus* cultured in PDA mediumhas been reported in earlier studies[33]. The influence of different culture media on biomass production of *A. terreus* in terms of mycelial dry weight is shown in figure 3a. *A. terreus* exhibited a profuse growth and highest metabolite production in the PDA broth when compared with the other tested media. Moderate growth and DPPH scavenging activity was observed in potato carrot broth, malt extract broth, Sabouraud dextrose broth, and Czapek's Dox broth, respectively. Thus, among different nutrient media, the PDA medium was found to be the best one for a high yield biomass production and exhibit antioxidant activity. The current observation is in parallel with the previous report[34], where PDB and Sabouraud's dextrose were found to be better nutrient media for growth and metabolite production of *A. terreus*. PDB has been shown to exhibitthe maximum results for the biomass and pigment production of *A. terreus* KMBF1501 in the study conducted by Akilandeswari and Pradeep[35].

Among all the tested carbon sources, sucrose was found to be the best carbon source for antioxidant metabolite production (Fig.4b, p<0.001), which was followed by cellulose and maltose. Biomass production was found to be high in the medium supplemented with cellulose followed by sucrose and dextrose (Fig 4a). Carboxy methylcellulose was the least utilized compound by the fungus.

Mathan et al.[30] and Bhattacharyya & Jha[36] reported a profuse growth and maximal metabolite production by *Aspergillus* on all the carbon sources and sucrose-supplemented media. According to the reports by Arora and Chandra (2010)[37] on the assay of antioxidant potential of two *Aspergillus* isolates by different methods under various physicochemical conditions,higher activity was observed in sucrose as carbon source followed by dextrose and maltose which are in consonance with the present study. However, it is contradictory to the general concept that glucose and

starch are the best carbon source for fungal growth by Yen and Chang (1999)[32].

Fig (5) presents the results obtained for the effect of different nitrogen sources on biomass and antioxidant metabolite production. Maximal biomass production was noted in the culture medium supplemented with urea, followed by $(NH_4)_2SO_4$ and NH_4Cl. Statistically, all these exhibited a significant biomass production (mycelial dry weight) compared to the control ($p<0.001$). Yeast extract also showed a substantial high yield biomass production ($p<0.01$). With reference to the DPPH scavenging activity, supplementation of($(NH_4)_2SO_4$ and NH_4Cl, gave the maximum results ($p<0.001$), followed by the malt extract ($p<0.01$). It was noted that there was no clear-cut correlation between biomass production and antioxidant activity of the crude extract of the fungus. This indicates that the production of metabolites with potential antioxidant activity varies depending upon the supplemented nitrogen sources.

From the results, it was evident that the fungi exhibited growth at its maximal level and metabolite production in the acidic pH (Table 1). pH 5.5 was found to be the best for both metabolites and biomass production, but the fungal biomass and bioactive compound synthesis was found to be the least in the media with alkaline pH. The influence of temperature on mycelial growth and bioactive antioxidant metabolite production by *A. terreus* was presented in the table 1. The maximal growth and DPPH activity was observed at 28°C followed by 37°C. There was a decrease in both biomass and antioxidant bioactive production at 23°C and 42°C. The results obtained are in parallel with the earlier reports; incubation temperatures ranges from 25 to 30°C enhanced the growth and synthesis of bioactive fractions by certain *Aspergillus* species in a study carried out by Bhattacharyya and Jha[36] & Jain and Pundir[38]. *A. terreus* showed a profuse growth and biomass production at salinity 4 % to 6% with optimal growth at 4% (Table 1). The secondary metabolite production was higher at asalinity range of 5% to 7% with the best result at 7% NaCl concentration. The results were in parallel with the study conducted by Masuma *et al*.[6] on the effect of increasing salinity in seawater on the growth of certain *Aspergillus* and other marine fungi.

The results of the experiment carried out for the combined optimal conditions (28°C; pH 5.5 with 7% NaCl), indicated there was a positive changes. The yield of mycelial dry weight (253.6±3.92) was found to be significantly higher compared to the control ($p<0.001$). The DPPH scavenging activity (78.5202±1.78) was also found to be significantly higher at the level of $p<0.01$ compared to the controlled conditions. Thus, the optimized conditions such as temperature, pH, and salt concentration in addition to the PDB supplemented with sucrose and ammonium sulfate as carbon and nitrogen sources, provide ideal conditions for high-yield biomass production of secondary metabolites and free radical-scavenging activity from *A. terreus* in laboratories.

CONCLUSION:

From the present study, it is evident that *A. terreus* is a potent source of antioxidant compounds which can be exploited widely in pharmaceutical industries by employing different kinds of optimized physicochemical conditions. The findings of the present work suggest that the formulation of growth media and optimized physical and chemical parameters conditions will be helpful in the mass cultivation of the fungus for its industrial-scale production of bioactive molecules. Further studies like identification of bioactive compounds, statistical approaches for media optimization and gene expression mechanism of hyper activation of metabolite synthesis pathways is highly recommended for a better understanding of the downstream processing and economical production.

List of Symbols and Abbreviations:

A.*terreus*- *Aspergillus terreus*
C/N- carbon/nitrogen
CDA-Czepek Dox agar
CMC- carbodxy methyl cellulose
DPPH-1, 1-diphenyl-2-picryl-hydrazyl
MEA-malt Extract agar
PDA-potato dextrose agar
ROS -Reactive Oxygen Species
SDA-Sabouraud's dextrose agar
SmF –submerged fermentation
SSF-solid substrate fermentation

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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